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SPECIFICATION
NUCLEIC ACID PROBES FOR
DETECTION AND/OR QUANTITATION OF
NON-VIRAL ORGANISMS

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Continuation-In-Part of Hogan et al., App. Ser. No. 083,542 filed August 7, 1987, which is a Continuation-In-Part of Hogan et al., App. Ser. No. 934,244 filed November 24, 1986.

BACKGROUND OF THE INVENTION

10 1. Field of the Invention

The inventions described and claimed herein relate to probes and assays based on the use of genetic material such as RNA. More particularly, the inventions relate to the design and construction of nucleic acid probes and hybridization of such probes to genetic material of target non-viral organisms in assays for detection and/or quantitation thereof in test samples of, e.g., sputum, urine, blood and tissue sections, food, soil and water.

20 2. Introduction

Two single strands of nucleic acid, comprised of nucleotides, may associate ("hybridize") to form a double helical structure in which the two polynucleotide chains running in opposite directions are held together by hydrogen bonds (a weak form of chemical bond) between pairs of matched, centrally located compounds known as "bases." Generally, in the double helical structure of nucleic acids, for example, the base adenine

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(A) is hydrogen bonded to the base thymine (T) or uracil (U) while the base guanine (G) is hydrogen bonded to the base cytosine (C). At any point along the chain, therefore, one may find the base pairs AT or AU, TA or UA, GC, or CG. One may also
5 find AG and GU base pairs in addition to the traditional ("canonical") base pairs. Assuming that a first single strand of nucleic acid is sufficiently complementary to a second and that the two are brought together under conditions which will promote their hybridization, double stranded nucleic acid will result.
10 Under appropriate conditions, DNA/DNA, RNA/DNA, or RNA/RNA hybrids may be formed.

Broadly, there are two basic nucleic acid hybridization procedures. In one, known as "in solution" hybridization, both a
15 "probe" nucleic acid sequence and nucleic acid molecules from a test sample are free in solution. In the other method, the sample nucleic acid is usually immobilized on a solid support and the probe sequence is free in solution.

A probe may be a single strand nucleic acid sequence which is complementary in some particular degree to the nucleic
20 acid sequences sought to be detected ("target sequences"). It may also be labelled. A background description of the use of nucleic acid hybridization as a procedure for the detection of particular nucleic acid sequences is described in U.S. Application Serial No. 456,729, entitled "Method for Detection,
25 Identification and Quantitation of Non-Viral Organisms," filed January 10, 1983 (Kohne I), ^{now issued as U.S. Patent No. 4,851,330} and U.S. Application Serial No. 655,365, entitled "Method For Detecting, Identifying and Quantitating Organisms and Viruses," filed September 4, 1984
^{now issued as U.S. Patent No. 5,288,611} (Kohne II), both of which are incorporated by reference, together

with all other applications cited herein.

Also described in those applications are methods for determining the presence of RNA-containing organisms in a sample which might contain such organisms, comprising the steps of
5 bringing together any nucleic acids from a sample and a probe comprising nucleic acid molecules which are shorter than the rRNA subunit sequence from which it was derived and which are sufficiently complementary to hybridize to the rRNA of one or more non-viral organisms or groups of non-viral organisms,
10 incubating the mixture under specified hybridization conditions, and assaying the resulting mixture for hybridization of the probe and any test sample rRNA. The invention is described to include using a probe which detects only rRNA subunit subsequences which are the same or sufficiently similar in particular organisms or
15 groups of organisms and is said to detect the presence or absence of any one or more of those particular organisms in a sample, even in the presence of many non-related organisms.

We have discovered and describe herein a novel method and means for designing and constructing DNA probes for use in
20 detecting unique rRNA sequences in an assay for the detection and/or quantitation of any group of non-viral organisms. Some of the inventive probes herein may be used to detect and/or quantify a single species or strain of non-viral organism and others may be used to detect and/or quantify members of an entire genus or
25 desired phylogenetic grouping.

SUMMARY OF THE INVENTION

In a method of probe preparation and use, a single strand deoxyoligonucleotide of particular sequence and defined length is used in a hybridization assay to determine the presence or amount of rRNA from particular target non-viral organisms to distinguish them from their known closest phylogenetic neighbors. Probe sequences which are specific, respectively, for 16S rRNA variable subsequences of Mycobacterium avium, Mycobacterium intracellulare and the Mycobacterium tuberculosis-complex bacteria, and which do not cross react with nucleic acids from each other, or any other bacterial species or respiratory infectious agent, under proper stringency, are described and claimed. A probe specific to three 23S rRNA variable region subsequences from the Mycobacterium tuberculosis-complex bacteria is also described and claimed, as are rRNA variable region probes useful in hybridization assays for the genus Mycobacterium (16S 23S rRNA specific), Mycoplasma pneumoniae (5S and 16S rRNA-specific), Chlamydia trachomatis (16S and 23S rRNA specific), Enterobacter cloacae (23S rRNA specific), Escherichia coli (16S rRNA specific), Legionella (16S and 23S rRNA specific), Salmonella (16S and 23S rRNA specific), Enterococci (16S rRNA specific), Neisseria gonorrhoeae (^{16S}~~16S~~-rRNA specific), Campylobacter (16S rRNA specific), Proteus mirabilis (23S rRNA specific), Pseudomonas (23S rRNA specific), fungi (18S and 28S rRNA specific), and bacteria (16S and 23S rRNA specific).

In one embodiment of the assay method, a test sample is first subjected to conditions which release rRNA from any non-viral organisms present in that sample. rRNA is single stranded and therefore available for hybridization with

sufficiently complementary genetic material once so released. Contact between a probe, which can be labelled, and the rRNA target may be carried out in solution under conditions which promote hybridization between the two strands. The reaction mixture is then assayed for the presence of hybridized probe. Numerous advantages of the present method for the detection of non-viral organisms over prior art techniques, including accuracy, simplicity, economy and speed will appear more fully from the detailed description which follows.

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BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a chart of the primary structure of bacterial 16S rRNA for Escherichia coli, depicting standard reference numbers for bases.

15 Figure 2 is a chart of the primary structure of bacterial 23S rRNA for Escherichia coli, depicting standard reference numbers for bases.

Figure 3 is a chart of the primary structure of bacterial 5S rRNA for Escherichia coli, depicting standard reference numbers for bases.

20 Figure 4 is a chart of the primary structure for the 18S rRNA for Saccharomyces cerevisiae, depicting standard reference numbers for bases.

25 Figure 5 is a chart of the primary structure for the 28S rRNA for Saccharomyces cerevisiae, depicting standard reference numbers for bases.

Figure 6 is a diagram showing the locations in the 16S rRNA (using E. coli reference numbers) which differ between 12

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different sets of related organisms. In Example 1, for example,
99.7%
29.7 refers to the difference in 16s rRNA between Clostridium
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botulinum and Clostridium subterminale.

Figure 7 is a diagram showing the locations in the
5 first 1500 bases of 23S rRNA (using E.coli reference numbers)
which differ between 12 different sets of related organisms.

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Figure 8 is a diagram showing the locations in the
terminal bases of 23S rRNA (using E.coli reference numbers) which
differ between 12 different sets of related organisms.

10 Figure 9 is a schematic representation of the location
of probes capable of hybridizing to the 16S rRNA.

Figure 10 is a schematic representation of the location
of probes capable of hybridizing to the first 1500 bases of the
23S rRNA.

15 Figure 11 is a schematic representation of the location
of probes capable of hybridizing to the terminal bases of 23S
rRNA.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

20 The following terms, as used in this disclosure and
claims, are defined as:

nucleotide: a subunit of a nucleic acid consisting of
a phosphate group, a 5' carbon sugar and a nitrogen containing
base. In RNA the 5' carbon sugar is ribose. In DNA, it is a 2-
25 deoxyribose. The term also includes analogs of such subunits.

nucleotide polymer: at least two nucleotides linked by
phosphodiester bonds.

oligonucleotide: a nucleotide polymer generally about 10 to about 100 nucleotides in length, but which may be greater than 100 nucleotides in length.

5 nucleic acid probe: a single stranded nucleic acid sequence that will combine with a complementary single stranded target nucleic acid sequence to form a double-stranded molecule (hybrid). A nucleic acid probe may be an oligonucleotide or a nucleotide polymer.

10 hybrid: the complex formed between two single stranded nucleic acid sequences by Watson-Crick base pairings or non-canonical base pairings between the complementary bases.

hybridization: the process by which two complementary strands of nucleic acids combine to form double stranded molecules (hybrids).

15 complementarity: a property conferred by the base sequence of a single strand of DNA or RNA which may form a hybrid or double stranded DNA:DNA, RNA:RNA or DNA:RNA through hydrogen bonding between Watson-Crick base pairs on the respective strands. Adenine (A) usually complements thymine (T) or Uracil
20 (U), while guanine (G) usually complements cytosine (C).

stringency: term used to describe the temperature and solvent composition existing during hybridization and the subsequent processing steps. Under high stringency conditions only highly homologous nucleic acid hybrids will form; hybrids
25 without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determine the amount of complementarity needed between two nucleic acid strands forming a hybrid. Stringency is chosen to maximize the

difference in stability between the hybrid formed with the target and the nontarget nucleic acid.

probe specificity: characteristic of a probe which describes its ability to distinguish between target and non-target sequences. Dependent on sequence and assay conditions. Probe specificity may be absolute (i.e., probe able to distinguish between target organisms and any nontarget organisms), or it may be functional (i.e., probe able to distinguish between the target organism and any other organism normally present in a particular sample). Many probe sequences can be used for either broad or narrow specificity depending on the conditions of use.

variable region: nucleotide polymer which differs by at least one base between the target organism and nontarget organisms contained in a sample.

conserved region: a region which is not variable.

sequence divergence: process by which nucleotide polymers become less similar during evolution.

sequence convergence: process by which nucleotide polymers become more similar during evolution.

bacteria: members of the phylogenetic group eubacteria, which is considered one of the three primary kingdoms.

T_m: temperature at which 50% of the probe is converted from the hybridized to the unhybridized form.

thermal stability: Temperature at which 50% of the probe:target hybrids are converted to the single stranded form. Factors which affect the thermal stability can affect probe specificity and therefore, must be controlled. Whether a probe sequence is useful to detect only a specific type of organism

depends largely on the thermal stability difference between probe:target hybrids ("P:T") and probe:nontarget hybrids ("P:NT"). In designing probes the T_m P:T minus the T_m P:NT should be as large as possible.

5 In addition to a novel method for selecting probe sequences, we have discovered that it is possible to create a DNA probe complementary to a particular rRNA sequence obtained from a single type of target microorganism and to successfully use that probe in a non-cross reacting assay for the detection of that
10 single microorganism, even in the presence of its known, most closely related taxonomic or phylogenetic neighbors. With the exception of viruses, all prokaryotic organisms contain rRNA molecules including 5S rRNA, 16S rRNA, and a larger rRNA molecule known as 23S rRNA. Eukaryotes are known to have 5.0S, 5.8S, 18S
15 and 28S rRNA molecules or analogous structures. (The term "16S like" sometimes is used to refer to the rRNA found in the small ribosomal subunit, including 18S and 17S rRNA. Likewise the term "23S like" rRNA sometimes is used to refer to the rRNA found in the large ribosomal subunit. 5.8S rRNA is equivalent to the 5'
20 end of the 23S like rRNA.) These rRNA molecules contain nucleotide sequences which are highly conserved among all organisms thus far examined. There are known methods which allow a significant portion of these rRNA sequences to be determined. For example, complementary oligonucleotide primers of about 20-30
25 bases in length can be hybridized to universally conserved regions in purified rRNA that are specific to the 5S, 16S, or 23S subunits and extended with the enzyme reverse transcriptase. Chemical degradation or dideoxynucleotide- terminated sequencing reactions can be used to determine the nucleotide sequence of the

extended product. Lane, D.J. et al., Proc. Nat'l Acad. Sci. USA
82, 6955-6959 (1985).

5 In our invention, comparison of one or more sequenced
rRNA variable regions from a target organism to one or more rRNA
variable region sequences from a closely related bacterial
species is utilized to select a sequence unique to the rRNA of
the target organism. rRNA is preferable to DNA as a probe target
because of its relative abundance and stability in the cell and
because of its patterns of phylogenetic conservation.

10 Notwithstanding the highly conserved nature of rRNA, we
have discovered that a number of regions of the rRNA molecule
which can vary in sequence, can vary even between closely related
species and can, therefore, be utilized to distinguish between
such organisms. Differences in the rRNA molecule are not
15 distributed randomly across the entire molecule, but rather are
clustered into specific regions. The degree of conservation also
varies, creating a unique pattern of conservation across the
ribosomal RNA subunits. The degree of variation and the
distribution thereof, can be analyzed to locate target sites for
20 diagnostic probes. This method of probe selection may be used to
select more than one sequence which is unique to the rRNA of a
target organism.

We have identified variable regions by comparative
analysis of rRNA sequences both published in the literature and
25 sequences which we have determined ourselves using procedures
known in the art. We use a Sun Microsystems (TM) computer for
comparative analysis. The compiler is capable of manipulating
many sequences of data at the same time. Computers of this type
and computer programs which may be used or adapted for the

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these increased variation densities usually are situated in the same regions of the 16S and 23S rRNA for comparable per cent similarity values. In this manner, we have observed that certain regions of the 16S and 23S rRNA are the most likely sites in which significant variation exists between the target organism and the closest phylogenetic relatives found in a sample. We have disclosed and claimed species specific probes which hybridize in these regions of significant variation between the target organism and the closest phylogenetic relative found in a sample.

Figures 9, 10 and 11 are a schematic representation of the location of probes disclosed and claimed herein. Because 16S and 23S RNAs do not, as a rule, contain sequences of duplication longer than about six nucleotides in length, probes designed by these methods are specific to one or a few positions on the target nucleic acid.

The sequence evolution at each of the variable regions (for example, spanning a minimum of 10 nucleotides) is, for the most part divergent, not convergent. Thus, we can confidently design probes based on a few rRNA sequences which differ between the target organism and its phylogenetically closest relatives. Biological and structural constraints on the rRNA molecule which maintain homologous primary, secondary and tertiary structure throughout evolution, and the application of such constraints to probe diagnostics is the subject of ongoing study. The greater the evolutionary distance between organisms, the greater the number of variable regions which may be used to distinguish the organisms.

Once the variable regions are identified, the sequences

are aligned to reveal areas of maximum homology or "match". At this point, the sequences are examined to identify potential probe regions. Two important objectives in designing a probe are to maximize homology to the target sequence(s) (greater than 90% homology is recommended) and to minimize homology to non-target sequence(s) (less than 90% homology to nontargets is recommended). We have identified the following useful guidelines for designing probes with desired characteristics.

First, probes should be positioned so as to minimize the stability of the probe:nontarget nucleic acid hybrid. This may be accomplished by minimizing the length of perfect complementarity to non-target organisms, avoiding G and C rich regions of homology to non-target sequences, and by positioning the probe to span as many destabilizing mismatches as possible (for example, dG:rU base pairs are less destabilizing than some others).

Second, the stability of the probe: target nucleic acid hybrid should be maximized. This may be accomplished by avoiding long A and T rich sequences, by terminating the hybrids with G:C base pairs and by designing the probe with an appropriate T_m . The beginning and end points of the probe should be chosen so that the length and %G and %C result in a T_m about 2-10°C higher than the temperature at which the final assay will be performed. The importance and effect of various assay conditions will be explained further herein. Third, regions of the rRNA which are known to form strong structures inhibitory to hybridization are less preferred. Finally, probes with extensive self-complementarity should be avoided.

In some cases, there may be several sequences from a

particular region which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base.

5 The following chart indicates how, for one embodiment of the invention useful in the detection of a nucleic acid in the presence of closely related nucleic acid sequences, unique sequences can be selected. In this example, rRNA sequences have been determined for organisms A-E and their sequences,
10 represented numerically, are aligned as shown. It is seen that sequence 1 is common to all organisms A-E. Sequences 2-6 are found only in organisms A, B and C, while sequences 8, 9 and 10 are unique to organism A. Therefore, a probe complementary to sequences 8, 9 or 10 would specifically hybridize to organism A.

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Illustrative Pattern of Sequence Relationships Among Related Bacteria

<u>Organism</u>		<u>rRNA Sequence</u>									
20	A	1	2	3	4	5	6	7	8	9	10
	B	1	2	3	4	5	6	7	11	12	13
	C	1	2	3	4	5	6	14	15	16	17
	D	1	18	19	20	21	22	23	24	25	26
	E	1	18	19	20	21	27	28	29	30	31

25 In cases where the patterns of variation of a macromolecule are known, for example, rRNA, one can focus on specific regions as likely candidates for probe design. However, it is not always necessary to determine the entire nucleic acid sequence in order to obtain a probe sequence. Extension from any
30 single oligonucleotide primer can yield up to 300-400 bases of sequence. When a single primer is used to partially sequence the rRNA of the target organism and organisms closely related to the

target, an alignment can be made as outlined above. Plainly, if a useful probe sequence is found, it is not necessary to continue rRNA sequencing using other primers. If, on the other hand, no useful probe sequence is obtained from sequencing with a first
5 primer, or if higher sensitivity is desired, other primers can be used to obtain more sequences. In those cases where patterns of variation for a molecule are not well understood, more sequence data may be required prior to probe design.

Thus, in Examples 1-3 below, two 16S-derived primers
10 were used. The first primer did not yield probe sequences which met the criteria listed herein. The second primer yielded probe sequences which were determined to be useful following characterization and testing for specificity as described. In Example 4, six 23S primers were used prior to locating the probe
15 sequence set forth.

Once a presumptive unique sequence has been identified, a complementary DNA oligonucleotide is synthesized. This single stranded oligonucleotide will serve as the probe in the DNA/rRNA assay hybridization reaction. Defined oligonucleotides may be
20 synthesized by any of several well known methods, including automated solid-phase chemical synthesis using cyano-ethylphosphoramidite precursors. Barone, A.D. et al., Nucleic Acids Research 12, 4051-4060 (1984). In this method,
B deoxyoligonucleotides are synthesized on solid polymer supports.
25 Release of the oligonucleotide from the support is accomplished by treatment with ammonium hydroxide at 60°C for 16 hours. The solution is dried and the crude product is dissolved in water and separated on polyacrylamide gels which generally may vary from 10-20% depending upon the length of the fragment. The major

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band, which is visualized by ultraviolet back lighting, is cut from the gel with a razor blade and extracted with ^{0.1 M}~~0.1 M~~ ammonium acetate, pH 7.0, at room temperature for 8-12 hours. Following centrifugation, the supernatant is filtered through a 0.4 micron filter and desalted on a P-10 column (Pharmacia). Other well known methods for construction of synthetic oligonucleotides may, of course, be employed.

Current DNA synthesizers can produce large amounts of synthetic DNA. After synthesis, the size of the newly made DNA is examined by gel filtration and molecules of varying size are generally detected. Some of these molecules represent abortive synthesis events which occur during the synthesis process. As part of post-synthesis purification, the synthetic DNA is usually size fractionated and only those molecules which are the proper length are kept. Thus, it is possible to obtain a population of synthetic DNA molecules of uniform size.

It has been generally assumed, however, that synthetic DNA is inherently composed of a uniform population of molecules all of the same size and base sequence, and that the hybridization characteristics of every molecule in the preparation should be the same. In reality, preparations of synthetic DNA molecules are heterogeneous and are composed of significant numbers of molecules which, although the same size, are in some way different from each other and have different hybridization characteristics. Even different preparations of the same sequence can sometimes have different hybridization characteristics.

Accordingly, preparations of the same synthetic probe sequence can have different hybridization characteristics. Because

of this the specificity of probe molecules from different preparations can be different. The hybridization characteristics of each preparation should be examined in order to determine the hybridization conditions which must be used in order to obtain the desired probe specificity. For example, the synthetic probe described in Example 4 below has the specificity profile described in Table 14. This data was obtained by using the hybridization and assay conditions described. A separate preparation of this probe which has different hybridization characteristics may not have precisely the same specificity profile when assayed under the conditions presented in Example 4. Such probe preparations have been made. To obtain the desired specificity, these probes can be hybridized and assayed under different conditions, including salt concentration and/or temperature. The actual conditions under which the probe is to be used must be determined, or matched to extant requirements, for each batch of probe since the art of DNA synthesis is somewhat imperfect.

Following synthesis and purification of a particular oligonucleotide sequence, several procedures may be utilized to determine the acceptability of the final product. The first is polyacrylamide gel electrophoresis, which is used to determine size. The oligonucleotide is labelled using, for example, ^{32}P -ATP and T_4 polynucleotide kinase. The labelled probe is precipitated in ethanol, centrifuged and the dried pellet resuspended in loading buffer (80% formamide, 20 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The samples are heated for five minutes at 90°C and loaded onto a denaturing polyacrylamide gel. Electrophoresis is carried out in TBE buffer

(0.1 M Tris HCl pH 8.3, 0.08 M boric acid, 0.002 M EDTA) for 1-2 hours at 1,000 volts. Following electrophoresis of the oligonucleotide the gel is exposed to X-ray film. The size of the oligonucleotide is then computed from the migration of oligonucleotide standards run concurrently.

The sequence of the synthetic oligonucleotide may also be checked by labelling it at the 5' end with ^{32}P -ATP and T_4 polynucleotide kinase, subjecting it to standard chemical degradation techniques, Maxam, A.M. and Gilbert, W., Proc. Nat'l. Acad. Sci. USA 74, 560-564 (1980), and analyzing the products on polyacrylamide gels. Preferably, the nucleotide sequence of the probe is perfectly complementary to the previously identified unique rRNA sequence, although it need not be.

The melting profile, including the melting temperature (T_m) of the oligonucleotide/ rRNA hybrids should also be determined. One way to determine T_m is to hybridize a ^{32}P -labelled oligonucleotide to its complementary target nucleic acid at 50°C in 0.1 M phosphate buffer, pH 6.8. The hybridization mixture is diluted and passed over a ^{2 cm} ~~2 cm~~ hydroxyapatite column at 50°C. The column is washed with 0.1 M phosphate buffer, 0.02% SDS to elute all unhybridized, single-stranded probes. The column temperature is then dropped 15°C and increased in 5°C increments until all of the probe is single-stranded. At each temperature, unhybridized probe is eluted and the counts per minute (cpm) in each fraction determined. The number of cpm shown to be bound to the hydroxyapatite divided by the total cpm added to the column equals the percent hybridization of the probe to the target nucleic acid.

An alternate method for determining thermal stability

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higher G-C content will be stable at higher temperatures.

We have discovered that the length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly homologous base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, oligonucleotide probes preferred in this invention are between about 15 and about 50 bases in length and are at least about 75-100% homologous to the target nucleic acid. For most applications 95-100% homology to the target nucleic acid is preferred.

Ionic strength and incubation temperature should also be taken into account in constructing a probe. It is known that the rate of hybridization will increase as ionic strength of the reaction mixture increases and that the thermal stability of hybrids will increase with increasing ionic strength. In general, optimal hybridization for synthetic oligonucleotide probes of about 15-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

As to nucleic acid concentration, it is known that the rate of hybridization is proportional to the concentration of the two interacting nucleic acid species. Thus, the presence of compounds such as dextran and dextran sulphate are thought to increase the local concentration of nucleic acid species and

thereby result in an increased rate of hybridization. Other agents which will result in increased rates of hybridization are specified in U.S. Application Serial No. 627,795, entitled "Accelerated Nucleic Acid Reassociation Method", filed July 5, 1984, Continuation-in-Part thereof, Serial No. (not yet assigned), filed June 4, 1987, and U.S. Application Serial No. 816,711, entitled "Accelerated Nucleic Acid Reassociation Method", filed January 7, 1986, both of which are incorporated by reference. ^{B²} On the other hand, chemical reagents which disrupt hydrogen bonds such as formamide, urea, DMSO, and alcohols will increase the stringency of hybridization.

Selected oligonucleotide probes may be labelled by any of several well known methods. Useful labels include radioisotopes as well as non-radioactive reporting groups. Isotopic labels include ³H, ³⁵S, ³²P, ¹²⁵I, Cobalt and ¹⁴C. Most methods of isotopic labelling involve the use of enzymes and include the known methods of nick translation, end labelling, second strand synthesis, and reverse transcription. When using radio-labelled probes, hybridization can be detected by autoradiography, scintillation counting, or gamma counting. The detection method selected will depend upon the hybridization conditions and the particular radioisotope used for labelling.

Non-isotopic materials can also be used for labelling, and may be introduced by the incorporation of modified nucleotides through the use of enzymes or by chemical modification of the probe, for example, by the use of non-nucleotide linker groups. Non-isotopic labels include fluorescent molecules, chemiluminescent molecules, enzymes, cofactors, enzyme substrates, haptens or other ligands. We

currently prefer to use acridinium esters.

In one embodiment of the DNA/rRNA hybridization assay invention, a labelled probe and bacterial target nucleic acids are reacted in solution. rRNA may be released from bacterial cells by the sonic disruption method described in Murphy, K.A. et al., U.S. Application Serial No. 841,860, entitled "Method for Releasing RNA and DNA From Cells", filed March 20, 1986, which is incorporated herein by reference. Other known methods for disrupting cells include the use of enzymes, osmotic shock, chemical treatment, and vortexing with glass beads. Following or concurrent with the release of rRNA, labelled probe may be added in the presence of accelerating agents and incubated at the optimal hybridization temperature for a period of time necessary to achieve significant reaction. Following this incubation period, hydroxyapatite may be added to the reaction mixture to separate the probe/rRNA hybrids from the non-hybridized probe molecules. The hydroxyapatite pellet is washed, recentrifuged and hybrids detected by means according to the label used.

Twenty-one embodiments illustrative of the claimed inventions are set forth below, in which a synthetic probe or probes complementary to a unique rRNA sequence from a target organism, or group of organisms is determined, constructed and used in a hybridization assay.

DESCRIPTION OF PARTICULAR EMBODIMENTS

Mycobacterium are acid-fast, alcohol fast, aerobic, non-mobile bacilli. Their lipid content is high and their growth slow. Mycobacterium avium and Mycobacterium intracellulare are together referred to as M. avium-intracellulare because they are

so difficult to differentiate. Recently, the M. avium complex, which includes M. intracellulare, was shown to be the second most commonly isolated, clinically significant Mycobacterium. Good, R.C. et al., J. Infect. Dis. 146, 829-833 (1982). More recent
5 evidence indicates that these organisms are a common cause of opportunistic infection in patients with AIDS (acquired immune deficiency syndrome). Gill, V.J. et al., J. Clin. Microbio. 22, 543-546 (1985). Treatment of such infections in AIDS patients is difficult because these organisms are resistant to most
10 antituberculosis drugs. Often a combination of five drugs are used in therapy. The severity of these infections also requires rapid diagnosis which, prior to the invention herein, was not available.

Members of the Mycobacterium tuberculosis complex (Mtb)
15 include Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum and Mycobacterium microti. The first three are pathogenic for humans while the last is an animal pathogen. These organisms produce slowly developing granulomas on the skin or they may invade internal organs. Tuberculosis of
20 the lungs can be disseminated to other parts of the body by the circulatory system, the lymph system, or the intestinal tract. Despite advances in public health and the advent of effective chemotherapy, Mycobacterial disease, tuberculosis in particular, continues to represent a major world-wide health problem.

25 The classical method for detecting bacteria in a test sample involves culturing of the sample in order to expand the number of bacterial cells present into observable colony growths which can be identified and enumerated. If desired, the cultures can also be subjected to additional testing in order to determine

antimicrobial susceptibility. Currently, the most widely used procedures for the detection, isolation and identification of Mycobacterium species are the acid-fast bacilli (AFB) smear (using either the Ziehl-Neelsen or fluorochrome techniques),
5 culture methods using Lowenstein-Jensen media and Middlebrook media, and biochemical tests. The AFB relies on the high lipid content of Mycobacterium to retain dye after exposure to acid-alcohol. While the AFB smear test is relatively rapid and simple to perform it does not always detect Mycobacteria and will not
10 differentiate between Mycobacterium avium and non-tuberculosis species, between Mycobacterium intracellulare and non-tuberculosis species, or between Mycobacterium tuberculosis-complex bacilli and non-tuberculosis species. For accurate identification of the infecting Mycobacterial species the
15 clinician must rely on culture results which can require anywhere from 3 to 8 weeks of growth followed by extensive biochemical testing. Other tests have been developed based on the detection of metabolic products from Mycobacterium using carbon-14 labelled substrates. In particular, the Bactec (TM) instrument can detect
20 the presence of Mycobacterium within 6 to 10 days of the time of inoculation. Gill, V.J., supra. However, the test does not distinguish Mycobacterium species. It is often important to make this determination so that particular drugs to which the organism is susceptible may be prescribed. For traditional culture
25 methods, this requires an additional 2 to 3 weeks and for the Bactec method, an additional 6 to 10 days.

In addition, specific embodiments for Mycoplasma pneumoniae, the Mycobacterium, Legionella, Salmonella, Chlamydia trachomatis, Campylobacter, Proteus mirabilis, Enterococcus,

Enterobacter cloacae, E. coli, Pseudomonas Group I, bacteria, fungi and Neisseria gonorrhoeae are set forth in the following examples.

As indicated by the below examples, the present invention has significant advantages over each of these prior art methods not only in the enhanced accuracy, specificity and simplicity of the test, but also in greatly reducing the time to achieve a diagnosis. The invention makes possible a definitive diagnosis and initiation of effective treatment on the same day as testing.

Example 1

Described below is the preparation of a single strand deoxyoligonucleotide of unique sequence and defined length which is labelled and used as a probe in a solution hybridization assay to detect the presence of rRNA from Mycobacterium avium. This unique sequence is specific for the rRNA of Mycobacterium avium and does not significantly cross-react under the hybridization conditions of this Example, with nucleic acids from any other bacterial species or respiratory infectious agent, including the closely-related Mycobacterium intracellulare. This probe is able to distinguish the two species, notwithstanding an approximate 98% rRNA homology between the two species. In this Example, as well as in Examples 2 and 3, sequences for M. avium, M. tuberculosis complex, M. intracellulare and related organisms were obtained by using a specific primer to a highly conserved region in the 16S rRNA. The sequence of this primer, derived from E. coli rRNA, was 5'-GGC CGT TAC CCC ACC TAC TAG CTA AT-3'. 5 nanograms of primer was mixed with 1 microgram of each rRNA to

β be sequenced in the presence of ^{0.1M KCL and 20mM} ~~0.1M KCL and 20mM~~ Tris-HCl pH 8.3 in a final volume of 10 microliters. The reactions were heated 10 min. at 45°C and then placed on ice. 2.5 microliters of ^{35S} dATP and 0.5 microliters of reverse transcriptase were added.

5 The sample was aliquoted into 4 tubes, each tube containing either dideoxy A, G, T, or C. The concentrations of these nucleotides are set forth in Lane et al., supra. The samples were incubated at 40°C for 30 minutes, and were then precipitated in ethanol, centrifuged and the pellets ^{lyophilized} ~~lyophilized~~ dry. Pellets

β 10 were resuspended in 10 microliters formamide dyes (100% formamide, 0.1% bromphenol blue and 0.1% xylene cyanol), and loaded onto 80 cm 8% polyacrylamide gels. The gels were run at 2000 volts for 2-4 hours.

Thus, nucleotide sequences for the 16S rRNA of

15 Mycobacterium avium and what were considered to be its closest phylogenetic neighbors, Mycobacterium intracellulare and Mycobacterium tuberculosis, were determined by the method of Lane, D.J. et al., Proc. Nat. Acad. Sci. USA 82:6955 (1985). In addition to determining the rRNA sequences for the organisms

20 noted above, a spectrum of clinically significant Mycobacterium were also sequenced. These included M. fortuitum, M. scrofulaceum and M. chelonae. Selected members of several genera closely related to Mycobacterium were also sequenced, including Rhodococcus bronchialis, Corynebacterium xerosis and Nocardia

25 asteroides.

Partial rRNA sequences from the above organisms were aligned for maximum nucleotide homology, using commercially available software from Intelligenetics, Inc., 1975 El Camino Real West, Mountain View, California 94040-2216 (IFIND Program).

From this alignment, regions of sequence unique to Mycobacterium avium were determined. The probe was selected so that it was perfectly complementary to a target nucleic acid sequence and so that it had a 10% or greater mismatch with the aligned rRNA from its known closest phylogenetic neighbor. A sequence 38 bases in length was chosen. The number of mismatched bases relative to the Mycobacterium avium sequence were as follows: Mycobacterium tuberculosis (8); Mycobacterium intracellulare (5); Mycobacterium scrofulaceum (6); Mycobacterium chelonae (12); and Mycobacterium fortuitum (10).

The following cDNA sequence was characterized by the criteria of length, T_m, and sequence analysis as described at pages 7-8 above and was determined to be specific for the rRNA Mycobacterium avium:

ACCGCAAAAGCTTTCCACCAGAAGACATGCGTCTTGAG.

This sequence is complementary to a unique segment found in the 16S rRNA of Mycobacterium avium. The size of the probe is 38 bases. The probe has a T_m of 74°C and sequence analysis by the method of Maxam & Gilbert (1980), *supra*, confirmed that the probe was correctly synthesized. The probe is capable of hybridizing to rRNA of M. avium in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for Mycobacterium avium, it was tested as a probe in hybridization reactions under the following conditions. ³²P-end-labeled oligonucleotide probes were mixed with 1 microgram (7x10⁻¹³ moles) of purified rRNA from Mycobacterium avium and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na₂HPO₄ and

NaH₂PO₄), 1 mM EDTA and 0.02% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer both with and without target present. Following separation on
5 hydroxyapatite as outlined in the patent applications identified at page 2, supra, the hybrids were quantitated by scintillation counting. These results are presented in Table 1, showing that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

TABLE 1

HYBRIDIZATION OF THE *M. AVIUM* PROBE
TO HOMOLOGOUS TARGET rRNA*

	<u>plus rRNA</u>	<u>minus rRNA</u>
<i>M. avium</i> probe	85-95%	0.5%

* %Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$

Specificity of the probe for *M. avium* was tested by mixing the ^{32}P labeled probe with rRNA released from cells of 29 other species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. ^{Patent 5,374,522} ~~Application Serial No. 841,860~~. 1×10^8 cells were suspended in 0.1 ml 5% SDS and sonicated for 10 minutes at 50-60°C. 1.0 ml of hybridization buffer (45% sodium diisobutyl sulfosuccinate, 40 mM phosphate buffer pH 6.8 and 1 mM EDTA) was added and the mixture incubated for 60 minutes at 72°C. Following incubation, 4.0 ml of hydroxyapatite solution (^{0.14 M} ~~0.14 M~~ sodium phosphate buffer, pH 6.8, 0.02% SDS and 1.0 gram hydroxyapatite per 50 mls solution) was added and incubated for 5 minutes at 72°C. The sample was centrifuged and the supernatant removed. 4.0 ml wash solution (0.14 M sodium phosphate pH 6.8) was added and sample was vortexed, centrifuged and the supernatant removed. The radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 2 and indicate that the probe is specific for *Mycobacterium avium* and does not react with any other mycobacterial species, including *Mycobacterium intracellulare*.

TABLE 2
HYBRIDIZATION OF THE M. AVIUM PROBE TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
5	Mycobacterium africanum	25420	1.0
	M. asiaticum	25276	1.2
	M. avium	25291	87.6
	M. bovis	19210	1.2
	M. bovis (BCG)	19015	1.0
10	M. chelonae	14472	0.9
	M. flavescens	14474	0.9
	M. fortuitum	6841	1.0
	M. gastri	15754	1.2
	M. gordonae	14470	1.2
15	M. haemophilum	29548	1.3
	M. intracellulare	13950	1.5
	M. kansasii	12478	1.2
	M. malmoense	29571	1.2
	M. marinum	827	1.2
20	M. nonchromogenicum	1930	1.1
	M. phlei	11758	1.3
	M. scrofulaceum	19981	1.2
	M. shimoidei	27962	2.3
	M. simiae	25275	1.2
25	M. smegmatis	el4468	1.0
	M. szulgai	23069	1.0
	M. terrae	15755	1.2
	M. thermoresistibile	19527	1.3
	M. triviale	23292	1.2
30	M. tuberculosis (avirulent)	25177	1.4
	M. tuberculosis (virulent)	27294	1.1
	M. ulcerans	19423	1.4
	M. vaccae	15483	1.2
35	M. xenopi	19971	1.5

As shown in Table 3 the probe also did not react with the rRNA from any of the respiratory pathogens which were also tested by the method just described. Nor did the probe react with any other closely related or phylogenetically more diverse species of bacteria also tested by that method (Table 4).

TABLE 3

HYBRIDIZATION OF M. AVIUM PROBE TO
RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
5	Corynebacterium xerosis	373	0.7
	Fusobacterium nucleatum	25586	1.3
	Haemophilum influenzae	19418	1.3
	Klebsiella pneumoniae	23357	1.8
10	Legionella pneumophila	33152	0.0
	Mycoplasma pneumoniae	15531	3.0
	Neisseria meningitidis	13090	0.0
	Pseudomonas aeruginosa	25330	0.0
	Propionibacterium acnes	6919	1.1
15	Streptococcus pneumoniae	6306	0.0
	Staphylococcus aureus	25923	1.5

TABLE 4

HYBRIDIZATION OF THE M. AVIUM PROBE TO A PHYLOGENETIC
CROSS SECTION OF BACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
20	Acinetobacter calcoaceticus	33604	0.0
	Branhamella catarrhalis	25238	0.6
	Bacillus subtilis	6051	0.9
25	Bacteroides fragilis	23745	1.0
	Campylobacter jejuni	33560	0.4
	Chromobacterium Violaceum	29094	1.7
	Clostridium perfringens	13124	2.1
	Deinococcus radiodurans	35073	0.8
30	Derxia gummosa	15994	0.3
	Enterobacter aerogenes	13048	0.6
	Escherichia coli	11775	0.3
	Mycobacterium gordonae	14470	1.9
	Mycoplasma hominis	14027	3.3
35	Proteus mirabilis	29906	0.0
	Pseudomonas cepacia	11762	1.0
	Rahnella aquatilis	33071	2.1
	Rhodospirillum rubrum	11170	0.6
	Streptococcus mitis	9811	0.9
40	Vibrio parahaemolyticus	17802	1.2
	Yersinia enterocolitica	9610	0.4

Example 2

After the alignment described in Example 1, the following sequence was characterized by the aforementioned criteria of length, T_m and sequence analysis and was determined to be specific for Mycobacterium intracellulare:

ACCGCAAAAGCTTTCCACCTAAAGACATGCGCCTAAAG

The sequence is complementary to a unique segment found in the 16S rRNA of Mycobacterium intracellulare. The size of the probe was 38 bases. The probe has a T_m of 75°C and sequence analysis confirmed that the probe was correctly synthesized. The probe hybridizes to RNA of M. intracellulare in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for the Mycobacterium intracellulare, the probe was tested in hybridization reactions under the following conditions. ^{32}P -end-labelled oligonucleotide probe was mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from Mycobacterium intracellulare and reacted in 0.12 M PB (equimolar amounts of $\overset{Na_2HPO_4}{\underset{\wedge}{Na_2HPO_4}}$ and $\overset{NaH_2PO_4}{\underset{\wedge}{NaH_2PO_4}}$), 1 mM EDTA and 0.2% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target Mycobacterium intracellulare rRNA present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. These results are shown in Table 5.

TABLE 5
HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO HOMOLOGOUS TARGET rRNA*

	<u>plus rRNA</u>	<u>minus rRNA</u>
M. <u>intracellulare</u> probe	85-95%	0.5%
* % Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$		

These data shows that the probe has a high extent of reaction to its homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the Mycobacterium intracellulare probe was tested by mixing the ³²P labelled probe with rRNA released from cells from 29 other species of mycobacteria by sonic disruption techniques described in Murphy et. al. U.S. Patent 5,374,522 Application No. 841,860. All hybridization assays were carried out as described in Example 1. Table 6 indicates that the probe is specific for Mycobacterium intracellulare and does not react with any other mycobacterial species, including Mycobacterium avium. These results are impressive in view of the 98% rRNA homology to M. avium; 98% homology to M. kansasii; 98% homology to M. asiaticum; and 97% homology to M. tuberculosis.

TABLE 6
HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Mycobacterium africanum	25420	0.9
	M. asiaticum	25276	1.1
	M. avium	25291	1.3
	M. bovis	19210	1.1

	M. bovis (BCG)	19015	1.2
	M. chelonae	14472	1.0
	M. favescent	14474	1.2
	M. fortuitum	6841	1.3
5	M. gastri	15754	1.3
	M. gordonae	14470	1.3
	M. haemophilum	29548	0.9
	M. intracellulare	13950	78.8
	M. kansasii	12479	1.1
10	M. Malmoense	29571	1.0
	M. marinum	827	0.9
	M. nonchromogenicum	1930	1.0
	M. phlei	11758	1.1
	M. scrofulaceum	19981	1.0
15	M. shimoidei	27962	1.3
	M. simiae	25275	1.1
	M. smegmatis	e14468	1.3
	M. szulgai	23069	1.0
	M. terrae	15755	1.4
20	M. thermoresistibile	19527	1.6
	M. triviale	23292	1.3
	M. tuberculosis (avirulent)	25177	1.2
	M. tuberculosis (virulent)	27294	1.2
	M. ulcerans	19423	1.1
25	M. vaccae	15483	1.0
	M. xenopi	19971	1.2

As shown in Table 7 the probe did not react with the rRNA from any of the respiratory pathogens tested in the hybridization assay. Nor did the probe react with any other closely related or phylogenetically more diverse species of bacteria that were tested (Table 8).

TABLE 7

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO RESPIRATORY PATHOGENS

	Organism	ATCC#	% Probe Bound
	Corynebacterium xerosis	373	2.2
	Fusobacterium nucleatum.	25586	1.5
40	Haemophilum influenzae	19418	1.3
	Klebsiella pneumoniae	23357	1.2
	Legionella pneumophila	33152	1.2
	Mycoplasma pneumoniae	15531	3.2
	Neisseria meningitidis	13090	1.1
45	Pseudomonas aeruginosa	25330	1.0
	Propionibacterium acnes	6919	2.9
	Streptococcus pneumoniae	6306	1.6
	Staphylococcus aureus	25923	1.3

TABLE 8
HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	Organism	ATTC#	% Probe
5	Acinetobacter calcoaceticus	33604	1.5
	Branhamella catarrhalis	25238	1.8
	Bacillus subtilis	6051	1.7
	Bacteroides fragilis	23745	1.9
10	Campylobacter jejuni	33560	1.9
	Chromobacterium Violaceum	29094	1.4
	Clostridium perfringens	13124	2.1
	Deinococcus radiodurans	35073	2.1
	Derxia gummosa	15994	1.6
15	Enterobacter aerogenes	13048	1.3
	Escherichia coli	11775	1.2
	Mycobacterium gordonae	14470	2.3
	Mycoplasma hominis	14027	2.6
	Proteus mirabilis	29906	1.2
20	Pseudomonas cepacia	11762	1.7
	Rahnella aquatilis	33071	1.5
	Rhodospirillum rubrum	11170	1.4
	Strptococcus mitis	9811	1.4
	Vibrio parahaemolyticus	17802	2.5
25	Yersinia enterocolitica	9610	1.1

Example 3

After the alignment described in Example 1, the following sequence was characterized by the aforementioned three criteria of size, sequence and T_m, and was determined to be specific to the Mtb complex of organisms, Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, and Mycobacterium microti:

1. TAAAGCGCTTTCCACCACAAGACATGCATCCCGTG.

The sequence is complementary to a unique segment found in the 16S rRNA of the Mtb-complex bacteria. The size of the probe is 35 bases. The probe has a T_m of 72°C and sequence analysis confirmed that the probe was correctly synthesized. It is capable of hybridizing in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for the Mtb complex the probe was tested in hybridization reactions under the following conditions. ^{32}P -end-labelled oligonucleotide probe was mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from Mycobacterium tuberculosis and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na_2HPO_4 , and NaH_2PO_4), 1 mM EDTA and 0.2% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target rRNA from Mycobacterium tuberculosis present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. The results are shown in Table 9.

TABLE 9

HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA PROBE
TO HOMOLOGOUS TARGET rRNA*

	plus rRNA	minus rRNA
Mtb complex probe	85-95%	0.5%

* % Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$

This data shows that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the probe for the Mtb complex was tested by mixing the ^{32}P labelled probe with rRNA released from cells of the 4 Mtb complex bacilli and of 25 other mycobacterial species by sonic disruption techniques described in Murphy et. al., U.S. Patent Application No. 841,860. All hybridization assays were

carried out as described in Example 1. Table 10 indicates that the probe is specific for organisms within the Mtb complex and does not react with any other mycobacterial species.

5

TABLE 10

HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA PROBE
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Mycobacterium africanum	25420	68.1
10	M. asiaticum	25276	3.4
	M. avium	25291	0.9
	M. bovis	19210	63.1
	M. chelonae	14472	1.1
	M. flavescens	14474	0.9
15	M. fortuitum	6841	1.1
	M. gastri	15754	0.8
	M. gordonae	14470	1.1
	M. haemophilum	29548	0.8
	M. intracellulare	13950	1.1
20	M. kansasii	12479	1.3
	M. malmoense	29571	0.9
	M. marinum	827	1.1
	M. nonchromogenicum	1930	1.1
	M. phlei	11758	1.3
25	M. scrofulaceum	19981	1.1
	M. shimoides	27962	1.0
	M. simiae	25275	1.2
	M. smegmatis	el4468	0.9
	M. szulgai	23069	1.1
30	M. terrae	15755	1.0
	M. thermoresistibile	19527	1.0
	M. triviale	23292	1.2
	M. tuberculosis (avirulent)	25177	66.2
	M. tuberculosis (virulent)	27294	62.4
35	M. ulcerans	19423	0.9
	M. vaccae	15483	0.8
	M. xenopi	19971	2.6

As shown in Table 11 the probe did not react with the
40 rRNA from any of the respiratory pathogens tested in the
hybridization assay. Nor did the probe react with any other
closely related or phylogenetically more diverse species of
bacteria that were tested (Table 12).

TABLE 11
HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA PROBE
TO RESPIRATORY PATHOGENS

5	Organism	ATCC#	% Probe Bound
	<i>Corynebacterium xerosis</i>	373	1.3
	<i>Fusobacterium nucleatum</i>	25586	1.0
	<i>Haemophilum influenzae</i>	19418	1.6
	<i>Klebsiella pneumoniae</i>	23357	1.2
10	<i>Legionella pneumophila</i>	33152	1.4
	<i>Mycoplasma pneumoniae</i>	15531	1.1
	<i>Neisseria meningitidis</i>	13090	1.0
	<i>Pseudomonas aeruginosa</i>	25330	1.7
	<i>Propionibacterium acnes</i>	6919	1.2
15	<i>Streptococcus pneumoniae</i>	25923	0.9

TABLE 12
HYBRIDIZATION OF THE Mtb-COMPLEX 16S rRNA DNA PROBE
TO A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

20	Organism	ATCC#	% Probe
	<i>Acinetobacter calcoaceticus</i>	33604	1.3
	<i>Branhamella catarrhalis</i>	25238	1.5
	<i>Bacillus subtilis</i>	6051	1.3
	<i>Bacteroides fragilis</i>	23745	1.3
25	<i>Campylobacter jejuni</i>	33560	1.1
	<i>Chromobacterium violaceum</i>	29094	1.0
	<i>Clostridium perfringens</i>	13124	1.2
	<i>Deinococcus radiodurans</i>	35073	1.0
	<i>Derxia gummosa</i>	15994	1.0
30	<i>Enterobacter aerogenes</i>	13048	1.0
	<i>Escherichia coli</i>	11775	1.0
	<i>Mycobacterium gordonae</i>	14470	1.3
	<i>Mycoplasma hominis</i>	14027	0.5
	<i>Proteus mirabilis</i>	29906	1.0
35	<i>Pseudomonas cepacia</i>	11762	2.6
	<i>Rahnella aquatilis</i>	33071	1.9
	<i>Rhodospirillum rubrum</i>	11170	1.0
	<i>Streptococcus mitis</i>	9811	1.1
	<i>Vibrio parahaemolyticus</i>	17802	0.9
40	<i>Yersinia enterocolitica</i>	9610	1.1

Two derivatives of the probe of Example 3 (numbered 2-3 below) were made and tested:

2. CCGCTAAAGCGCTTTCCACCACAAGACATGCATCCCCG

3. ACACCGCTAAAGCGCTTTCCACCACAAGACATGCATC.

All three probes have similar Tms (^{72.3°C}~~72.0~~; ^{73.5°C}~~73.5~~; and ^{72.3°C}~~72.0~~, respectively) and similar hybridization characteristics.

Hybridization to Mycobacterium tuberculosis complex organisms was 68-75% and non-specific hybridization to hydroxyapatite was less than 2%. Results of hybridization assay tests for these derivatives follow.

TABLE 13

HYBRIDIZATION OF PROBE OF EXAMPLES 3 AND 2
DERIVATIVES THEREOF
TO MYCOBACTERIAL SPECIES

Organism	ATCC#	Example % Probe 1 Bound	% Probe 2 Bound	% Probe 3 Bound
Mycobacterium africanum	25420	68.1	69.4	70.6
M. asiaticum	25274	3.4	5.3	1.8
M. avium	25291	0.9	1.6	1.4
M. bovis	19210	63.1	75.3	74
M. chelonae	14472	1.1	1.5	1.6
M. flavescens	14474	0.9	2.7	1.4
M. fortuitum	6841	1.1	3.6	1.5
M. gastri	15754	0.8	3.6	1.7
M. gordonae	14470	1.1	1.6	1.4
M. haemophilum	29548	0.8	3.2	1.7
M. intracellulare	13950	1.1	1.6	1.4
M. kansasii	12478	1.3	2.1	2.0
M. malmoense	29571	0.9	2.8	1.5
M. marinum	827	1.1	2.1	1.5
M. nonchromogenicum	1930	1.1	3.0	1.5
M. phlei	11758	1.3	1.3	1.1
M. scrofulaceum	19981	1.1	3.4	1.6
M. shimoides	27962	1.0	2.7	1.6
M. simiae	25275	1.2	2.9	1.8
M. smegmatis	e14468	0.9	1.5	1.2
M. szulgai	23069	1.1	3.6	1.1
M. terrae	15755	1.0	3.7	2.0
M. thermoresistibile	19527	1.0	1.6	1.3
M. triviale	23292	1.2	1.6	2.0
M. tuberculosis (avirulent)	25177	66.2	75	68
M. tuberculosis (virulent)	27294	62.4	74	75
M. ulcerans	19423	0.9	1.7	3.0
M. vaccae	15483	0.8	1.4	1.2
M. xenopi	19971	2.6	1.4	1.2

Example 4

The probe specific for the 23S rRNA of the M. tuberculosis complex was obtained by using a primer which was complementary to a highly conserved region of 23S rRNA. The sequence of this primer, derived from E. coli rRNA, was 5'-AGG AAC CCT TGG GCT TTC GG-3'. Five nanograms of this primer was mixed with 1 microgram of rRNA from M. tuberculosis and other closely related Mycobacterium and the procedure as described for Examples 1, 2 and 3 was followed. After alignment as described in Example 1, the following sequence was determined to be specific to the Mtb complex of organisms, Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, and Mycobacterium microti:

TGCCCTACCCACACCCACCACAAGGTGATGT.

The sequence is complementary to a unique segment found in the 23S rRNA of the Mtb-complex bacteria. The oligonucleotide probe was characterized as previously described by the criteria of length, T_m and sequence analysis. The size of the probe is 31 bases. The probe has a T_m of 72.5°C and sequence analysis confirmed that the probe was correctly synthesized. It is capable of hybridizing in the region corresponding to bases 1155-1190 of E. coli 23S rRNA.

To demonstrate the reactivity of this sequence for the Mtb complex the probe was tested in hybridization reactions under the following conditions. ^{32}P -end-labelled oligonucleotide probes were mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from Mycobacterium tuberculosis and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na_2HPO_4 , and NaH_2PO_4),

B 1 mM EDTA and ^{0.2%}~~0.2~~ SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target rRNA from Mycobacterium tuberculosis present.

5 Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. The results are shown in Table 14.

TABLE 14

10 HYBRIDIZATION OF THE Mtb-COMPLEX
23S rRNA DNA PROBE TO HOMOLOGOUS TARGET rRNA

	<u>plus rRNA</u>	<u>minus rRNA</u>
Mtb complex 23S probe	94%	1.2%

15 These data show that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the probe for the Mtb complex was tested by mixing the ³²P labelled probe with rRNA released from cells of the four Mtb complex bacilli and of 25 other mycobacterial species by sonic disruption techniques described in Murphy et al., U.S. Patent ^{5,374,522}~~Application No. 841,860~~. All hybridization assays were carried out as described in Example 1. Table 14 indicates that the probe is specific for organisms within the Mtb complex and does not react with any other mycobacterial species.

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TABLE 15
HYBRIDIZATION OF Mtb-COMPLEX 23S rRNA DNA PROBE
TO MYCOBACTERIAL SPECIES

	Organism	ATCC#	% Probe Bound
5	Mycobacterium africanum		
	M. asiaticum	25420	33.6
	M. avium	25276	1.2
	M. bovis	25291	1.0
10	M. chelonae	19210	32.0
	M. flavescens	14472	1.2
	M. fortuitum	14474	1.2
	M. gastri	6841	1.3
	M. gordonae	15754	1.1
15	M. haemophilum	14470	1.2
	M. intracellulare	29548	1.2
	M. kansasii	13950	1.1
	M. malmoense	12479	1.3
	M. marinum	29571	1.3
20	M. nonchromogenicum	827	1.2
	M. phlei	1930	1.0
	M. scrofulaceum	11758	1.0
	M. shimoidei	19981	1.1
	M. simiae	27962	1.2
25	M. smegmatis	25275	1.3
	M. szulgai	el4468	1.1
	M. terrae	23069	1.1
	M. thermoresistibile	15755	1.0
	M. triviale	19527	1.2
30	M. tuberculosis (avirulent)	23292	1.0
	M. tuberculosis (virulent)	25177	33.7
	M. ulcerans	27294	38.1
	M. vaccae	19423	1.3
35	M. xenopi	15483	1.0
		19971	1.3

Example 5

Three additional Mycobacterium tuberculosis complex probes, Examples 5-7 herein, were identified using two unique primers complementary to 23S rRNA. The first sequence is:

40 CCATCACCACCCTCCTCCGGAGAGGAAAAGG.

The sequence of this Example 5 was obtained using a 23S primer with the sequence 5'-GGC CAT TAG ATC ACT CC-3'. It was characterized and shown to be specific for the Mycobacterium tuberculosis complex of organisms including Mycobacterium

tuberculosis, Mycobacterium africanum and Mycobacterium bovis.

This sequence, from 23S rRNA, is 31 bases in length and has a T_m of 72°C. This probe is capable of hybridizing to RNA of the
aforementioned organisms in the region corresponding to bases
5 540-575 of E. coli 23S rRNA.

To demonstrate the reactivity and specificity of this
probe for Mycobacterium tuberculosis complex, it was tested as a
probe in hybridization reactions under the following conditions.
32P-end-labeled oligonucleotide probe was mixed with rRNA
10 released from cells of 30 species of mycobacteria by the sonic
disruption techniques described in Murphy et al., U.S. Patent
5,374,522
Application Serial No. 841,866. 3×10^7 cells were suspended in
0.1 ml 5% SDS and sonicated for 15 minutes at 50-60°C. One ml of
hybridization buffer (45% diisobutyl sulfosuccinate, 40 mM
15 phosphate buffer pH 6.8, 1 mM EDTA, 1 mM EGTA) was added and the
mixture incubated at 72°C for 2 hours. Following incubation, 4
ml of 2% (w/v) hydroxyapatite, ^{0.12 M}~~0.12 M~~ sodium phosphate buffer
^{pH 6.8}~~pH 6.8~~, 0.02% SDS, 0.02% sodium azide was added and incubated at
72°C for 5 minutes. The sample was centrifuged and the
20 supernatant removed. Four ml wash solution (^{0.12 M}~~0.12 M~~ sodium
phosphate buffer ^{pH 6.8}~~pH 6.8~~, 0.02% SDS, 0.02% sodium azide) was added
and the sample was vortexed, centrifuged and the supernatant
removed. The radioactivity bound to the hydroxyapatite was
determined by scintillation counting. The results are shown in
25 Table 16 and indicate that the probe is specific for the
Mycobacterium tuberculosis complex of organisms.

TABLE 16

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX
PROBE OF EXAMPLE 5 TO MYCOBACTERIAL SPECIES

5	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
	Mycobacterium africanum	25420	18.0
	M. asiaticum	25274	2.6
	M. avium	25291	3.4
	M. bovis	19210	21.7
10	M. bovis (BCG)	35734	35.3
	M. chelonae	14472	3.8
	M. flavescens	14474	2.3
	M. fortuitum	6841	1.8
	M. gastri	15754	2.2
15	M. gordonae	14470	2.8
	M. haemophilum	29548	2.8
	M. intracellulare	13950	2.1
	M. kansasii	12478	1.6
	M. malmoense	29571	2.3
20	M. marinum	827	2.1
	M. nonchromogenicum	1930	2.3
	M. phlei	11758	2.1
	M. scrofulaceum	19981	2.2
	M. shimoidei	27962	1.9
25	M. simiae	25275	2.2
	M. smegmatis	el4468	2.0
	M. szulgai	23069	2.2
	M. terrae	15755	2.2
	M. thermoresistibile	19527	2.2
30	M. triviale	23292	2.0
	M. tuberculosis (avirulent)	25177	26.4
	M. tuberculosis (virulent)	27294	36.6
	M. ulcerans	19423	2.5
	M. vaccae	15483	2.4
35	M. xenopi	19971	2.8

Table 16 shows that the probe also did not cross react with RNA from any of the closely related organisms tested by the method just described.

TABLE 17
HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX PROBE
OF EXAMPLE 5 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
5	Actinomadura madurae	19425	2.1
	Actinoplanes italicus	10049	3.1
	Arthrobacter oxidans	14358	2.1
	Brevibacterium linens	e9172	1.9
10	Corynebacterium xerosis	373	2.2
	Dermatophilus congolensis	14367	2.2
	Microbacterium lacticum	8180	2.1
	Nocardia asteroides	19247	2.0
	Nocardia brasiliensis	19296	2.2
15	Nocardia otitidis-caviarum	14629	2.0
	Nocardioopsis dassonvillei	23218	4.0
	Oerskovia turbata	33225	2.2
	Oerskovia xanthineolytica	27402	2.0
	Rhodococcus aichiensis	33611	1.9
20	Rhodococcus aurantiacus	25938	2.0
	Rhodococcus bronchialis	25592	2.1
	Rhodococcus chubuensis	33609	2.3
	Rhodococcus equi	6939	2.4
	Rhodococcus obuensis	33610	2.2
25	Rhodococcus sputi	29627	2.3

Example 6

The second Mycobacterium tuberculosis complex probe was obtained using a 23S primer with the sequence 5' CCT GAT TGC CGT
30 CCA GGT TGA GGG AAC CTT TGG G-3'. Its sequence is:

CTGTCCCTAAACCCGATTCAGGGTTCGAGGTTAGATGC

This sequence, from 23S rRNA, is 38 bases in length and has a T_m of 75°C. It hybridizes in the region corresponding to bases
2195-2235 of E. coli ^{23S} rRNA.

35 Like the complex probe in Example 5, this sequence was characterized and shown to be specific for the Mycobacterium tuberculosis complex of organisms including Mycobacterium tuberculosis, Mycobacterium africanum and Mycobacterium bovis.

To demonstrate the reactivity and specificity of the probe of this Example 6 to Mycobacterium tuberculosis complex, it was tested as a probe in hybridization reactions under the following conditions described for the probe in Example 5. The results are shown in Table 18 and indicate that the probe is specific for the Mycobacterium tuberculosis complex of organisms with the exception of Mycobacterium thermoresistibile, a rare isolate which is not a human pathogen.

10

TABLE 18

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX
PROBE OF EXAMPLE 6 TO MYCOBACTERIAL SPECIES

	Organism	ATCC #	% Probe Bound
	<u>Mycobacterium africanum</u>	25420	56.0
15	M. asiaticum	25274	3.1
	M. avium	25291	2.6
	M. bovis	19210	48.0
	M. bovis (BCG)	35734	63.0
	M. chelonae	14472	2.8
20	M. flavescens	14474	2.8
	M. fortuitum	6841	3.0
	M. gastri	15754	3.2
	M. gordonae	14470	3.0
	M. haemophilum	29548	3.0
25	M. intracellulare	13950	3.6
	M. kansasii	12478	3.9
	M. malmoense	29571	2.9
	M. marinum	827	2.9
	M. nonchromogenicum	1930	4.8
30	M. phlei	11758	2.9
	M. scrofulaceum	19981	2.6
	M. shimoides	27962	3.6
	M. simiae	25275	3.3
	M. smegmatis	el4468	3.0
35	M. szulgai	23069	2.8
	M. terrae	15755	2.8
	M. thermoresistibile	19527	11.7
	M. triviale	23292	3.2
	M. tuberculosis (avirulent)	25177	65.0
40	M. tuberculosis (virulent)	27294	53.0
	M. ulcerans	19423	2.5
	M. vaccae	15483	2.8
	M. xenopi	19971	3.3

Table 19 shows that the probe also did not cross react with RNA from any of the phylogenetically closely related organisms tested by the method just described.

5

TABLE 19

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX PROBE
OF EXAMPLE 6 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	Organism	ATCC #	% Probe Bound
10	Actinomadura madurae	19425	1.3
	Actinoplanes italicus	10049	0.6
	Arthrobacter oxidans	14358	1.1
	Brevibacterium linens	e9172	0.8
	Corynebacterium xerosis	373	1.0
15	Dermatophilus congolensis	14367	0.6
	Microbacterium lacticum	8180	1.9
	Nocardia asteroides	19247	0.9
	Nocardia brasiliensis	19296	0.8
	Nocardia otitidis-caviarum	14629	1.5
20	Nocardioptosis dassonvillei	23218	0.5
	Oerskovia turbata	33225	0.3
	Oerskovia xanthineolytica	27402	0.8
	Rhodococcus aichiensis	33611	1.6
	Rhodococcus aurantiacus	25938	0.7
25	Rhodococcus bronchialis	25592	1.5
	Rhodococcus chubuensis	33609	0.8
	Rhodococcus equi	6939	0.3
	Rhodococcus obuensis	33610	0.8
30	Rhodococcus sputi	29627	1.4

Example 7

The following additional Mycobacterium tuberculosis complex probe also has been identified using a 23S primer with the same sequence as that of Example 6, namely, 5'-CCT GAT TGC
35 CGT CCA GGT TGA GGG AAC CTT TGG G-3':

AGGCACTGTCCCTAAACCCGATTCAGGGTTC.

This sequence, from 23S rRNA is 31 bases in length and has a T_m of 71°C. It hybridizes in the region corresponding to bases 2195-2235 of E. coli 23S rRNA. As is the case with the

Mycobacterium tuberculosis complex probes of Examples 5 and 6 herein, this sequence also was characterized and shown to be specific for the Mycobacterium tuberculosis complex of organisms, including Mycobacterium tuberculosis, Mycobacterium africanum and Mycobacterium bovis.

To demonstrate the reactivity and specificity of this probe for Mycobacterium tuberculosis complex, it was tested as a probe in hybridization reactions under the conditions described for the probe of Example 5. Table 20 shows that the probe is specific for the Mycobacterium tuberculosis complex of organisms.

TABLE 20

HYBRIDIZATION OF THE MYCOBACTERIUM TUBERCULOSIS
COMPLEX PROBE OF EXAMPLE 7 TO MYCOBACTERIAL SPECIES

	Organism	ATCC #	% Probe Bound
15	<u>Mycobacterium africanum</u>	25420	43.0
	<u>M. asiaticum</u>	25274	0.6
	<u>M. avium</u>	25291	0.7
	<u>M. bovis</u>	19210	43.0
20	<u>M. bovis (BCG)</u>	35734	46.0
	<u>M. chelonae</u>	14472	0.6
	<u>M. flavescens</u>	14474	0.6
	<u>M. fortuitum</u>	6841	0.5
	<u>M. gastri</u>	15754	0.9
	<u>M. gordonae</u>	14470	0.7
25	<u>M. haemophilum</u>	29548	0.6
	<u>M. intracellulare</u>	13950	0.6
	<u>M. kansasii</u>	12478	0.9
	<u>M. malmoense</u>	29571	0.8
30	<u>M. marinum</u>	827	0.7
	<u>M. nonchromogenicum</u>	1930	0.8
	<u>M. phlei</u>	11758	0.6
	<u>M. scrofulaceum</u>	19981	0.7
	<u>M. shimoidei</u>	27962	0.8
35	<u>M. simiae</u>	25275	0.7
	<u>M. smegmatis</u>	e14468	0.6
	<u>M. szulgai</u>	23069	0.6
	<u>M. terrae</u>	15755	0.7
	<u>M. thermoresistibile</u>	19527	0.9
40	<u>M. triviale</u>	23292	0.7
	<u>M. tuberculosis (avirulent)</u>	25177	40.0

M. tuberculosis (virulent)	27294	50.0
M. ulcerans	19423	0.7
M. vaccae	15483	0.4
M. xenopi	19971	0.6

Table 21 shows that the probe also did not cross react with RNA from any of the closely related organisms tested by the method just described.

TABLE 21

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX PROBE OF
EXAMPLE 7 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

Organism	ATCC #	% Probe Bound
Actinomadura madurae	19425	1.0
Actinoplanes italicus	10049	0.6
Arthrobacter oxidans	14358	0.4
Brevibacterium linens	e9172	0.8
Corynebacterium xerosis	373	0.6
Dermatophilus congolensis	14367	0.8
Microbacterium lacticum	8180	0.5
Nocardia asteroides	19247	0.7
Nocardia brasiliensis	19296	0.5
Nocardia otitidis-caviarum	14629	0.6
Nocardiosis dassonvillei	23218	0.6
Oerskovia turbata	33225	0.8
Oerskovia xanthineolytica	27402	0.6
Rhodococcus aichiensis	33611	0.7
Rhodococcus aurantiacus	25938	0.7
Rhodococcus bronchialis	25592	0.6
Rhodococcus chubuensis	33609	0.6
Rhodococcus equi	6939	0.6
Rhodococcus obuensis	33610	0.6
Rhodococcus sputi	29627	0.9

Notably, overlapping probes may have identical specificity. Compare, for example, the probes of Examples 6 and 7:

Ex. 6 CTGTCCCTAAACCCGATTCAGGGTTCGAGGTTAGATGC

Ex. 7 AGGCACTGTCCCTAAACCCGATTCAGGGTTC

There may be several sequences from a particular region which will yield probes with the desired hybridization

characteristics. In other cases, one probe sequence may be significantly better than another probe differing by a single base. In general, the greater the sequence difference (% mismatch) between a target and nontarget organism, the more likely one will be able to alter the probe without affecting its usefulness for a specific application. This phenomenon also was demonstrated by the derivative probes in Example 3.

In Example 7, five bases were added to the 5' end of the probe in Example 6, and 12 bases were removed from the 3' end. The two probes have essentially identical hybridization characteristics.

Example 8

The Mycobacterium genus is particularly difficult to distinguish from Nocardia, Corynebacterium and Rhodococcus. These genera have common antigens, precipitins and G & C counts. Despite the fact that these organisms also exhibit 92-94% rRNA homology to the above listed Mycobacterium organisms, we have designed probes which detect all members of the genus Mycobacterium without cross reacting to the related genera.

In addition to the Mycobacterium species probes already disclosed, four probes specific for members of the Mycobacterium genus were identified using one primer complementary to 16S rRNA and one primer complementary to 23S rRNA. Sequence 1 was obtained using a 16S primer with the sequence 5'-TTA CTA GCG ATT CCG ACT TCA-3'. Sequences 2, 3 and 4 were obtained using a 23S primer with the sequence 5'-GTG TCG GTT TTG GGT ACG-3'. Sequence 1 is capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1025-1060 of E. coli 16S rRNA.

Sequences 2-4 hybridize in regions corresponding to the following bases of *E. coli* 23S rRNA in our numbering system (See Figure 2); 1440-1475; 1515-1555; 1570-1610 in our numbering system.

The following sequences were characterized and shown to be specific for the genus Mycobacterium:

1. CCA TGC ACC ACC TGC ACA CAG GCC ACA AGG
2. GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG
3. CAC CGA ATT CGC CTC AAC CGG CTA TGC GTC ACC TC
4. GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC

Sequence 1, from 16S rRNA, is 30 bases in length and has a T_m of 73°C . Sequence 2, from 23S rRNA, is 33 bases in length and has a T_m of 75°C . Sequence 3, from 23S rRNA, is 35 bases in length and has a T_m of 76°C . Sequence 4, from 23S rRNA, is 33 bases in length and has a T_m of 73°C .

To demonstrate the reactivity and specificity of probe 1 for members of the genus Mycobacterium, it was tested as a probe in hybridization reactions under the following conditions. ^{125}I -labeled oligonucleotide probe was mixed with rRNA released from cells of 30 species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. Patent Application ~~5,374,522~~ ^{5,374,522} Serial No. 841,868. 3×10^7 cells were suspended in 0.1 ml 5% SDS and sonicated for 15 minutes at $50-60^\circ\text{C}$. One ml of hybridization buffer (45% diisobutyl sulfosuccinate, 40 mM sodium phosphate ^{pH 6.8} ~~pH 6.8~~, 1 mM EDTA, 1 mM EGTA) was added and the mixture incubated at 72°C for 2 hours. Following incubation, 2 ml of separation solution (containing 2.5 g/l cationic magnetic microspheres, ^{0.17 M} ~~0.17 M~~ sodium phosphate buffer ^{pH 6.8} ~~pH 6.8~~, 7.5% Triton X-100 (TM), 0.02% sodium azide) was added and incubated at 72°C for 5 minutes. The RNA:probe hybrids, bound to the magnetic particles,

were collected and the supernatant removed. One ml wash solution
 0.12 M sodium phosphate buffer, pH 6.8, 14% diisobutyl
 sulfosuccinate, 5% Triton X-100, 0.02% sodium azide) was added,
 the particles collected and the supernatant removed. This step
 was repeated two times. The radioactivity bound to the magnetic
 particles was determined in a gamma counter. The results are
 shown in Table 22 and indicate that the probes hybridize to
 organisms in the genus Mycobacterium and that a combination of
 probes will detect all members of the genus. Table 23 shows that
 the probes do not react with other closely related bacteria.

TABLE 22
HYBRIDIZATION OF THE MYCOBACTERIUM
PROBES 1-4 TO MYCOBACTERIAL SPECIES

Organism	ATCC#	% Probe 1 Bound	% Probe 2 Bound	% Probe 3 Bound	% Probe 4 Bound
Mycobacterium africanum	25420	41.5	14.7	17.9	26.7
M. asiaticum	25274	31.8	20.2	7.9	0.1
M. avium	25291	11.7	34.7	10.1	1.6
M. bovis	19210	19.4	28.4	44.6	20.9
M. bovis (BCG)	35734	30.0	35.5	17.8	5.6
M. chelonae	14472	8.6	0.7	6.3	0.2
M. flavescens	14474	29.8	17.7	2.3	0.9
M. fortuitum	6841	34.7	2.2	4.8	0.2
M. gastri	15754	27.6	65.1	9.6	22.3
M. gordonae	14470	50.7	55.2	3.1	0.4
M. haemophilum	29548	40.7	60.7	0.4	12.4
M. intracellulare	13950	38.8	48.3	0.9	5.4
M. kansasii	12478	53.4	27.3	24.5	27.8
M. malmoense	29571	3.1	38.4	0.8	1.5
M. marinum	827	41.7	4.1	4.8	0.1
M. non-chromogenicum	1930	35.0	42.9	0.5	16.4
M. phlei	11758	23.7	0.6	1.8	0.6
M. scrofulaceum	19981	35.1	66.9	0.9	26.4
M. shimoides	27962	34.6	1.4	1.3	4.8
M. simiae	25275	45.9	44.0	5.3	0.1
M. smegmatis	14468	31.3	4.0	5.6	0.1
M. szulgai	23069	19.4	22.3	1.5	3.0
M. terrae	15755	25.6	21.7	0.4	12.3
M. thermo-resistibile	19527	20.3	34.5	3.1	17.6

5	M. triviale	23292	37.3	4.6	4.3	0.1
	M. tuberculosis (avirulent)	25177	38.5	26.3	11.3	23.0
	M. tuberculosis (virulent)	27294	13.8	12.4	38.4	22.3
	M. ulcerans	19423	33.9	28.7	0.4	8.9
	M. vaccae	15483	8.8	36.2	4.8	3.2
	M. xenopi	19971	38.4	2.1	3.8	0.2

10

TABLE 23

HYBRIDIZATION OF THE MYCOBACTERIUM PROBES
1-4 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe 1 Bound</u>	<u>% Probe 2 Bound</u>	<u>% Probe 3 Bound</u>	<u>% Probe 4 Bound</u>
15	Actinomadura madurae	19425	0.2	0.3	0.2	0.1
	Actinoplanes italicus	10049	0.4	0.5	0.3	0.2
20	Arthrobacter oxidans	14358	0.2	0.4	0.3	0.1
	Brevibacterium linens	e9172	0.3	0.3	0.3	0.1
	Corynebacterium xerosis	373	0.4	0.3	0.3	0.1
25	Dermatophilus congolensis	14367	0.4	0.6	0.3	0.2
	Microbacterium lacticum	8180	0.2	0.3	0.2	0.1
30	Nocardia asteroides	19247	0.3	0.3	0.4	0.1
	Nocardia brasiliensis	19296	0.4	0.3	0.6	0.1
	Nocardia otitidis- caviarum	14629	0.4	0.4	1.0	0.3
35	Nocardiopepsis dassonvillei	23218	0.3	0.2	0.3	0.1
	Oerskovia turbata	33225	0.2	0.2	0.3	0.1
40	Oerskovia xanthineolytica	27402	0.2	0.3	0.3	0.1
	Rhodococcus aichiensis	33611	0.4	0.2	0.3	0.2
45	Rhodococcus aurantiacus	25938	0.3	0.4	0.3	0.2
	Rhodococcus bronchialis	25592	0.4	0.3	0.3	0.1
	Rhodococcus chubuensis	33609	0.6	0.4	0.3	0.3

Rhodococcus equi	6939	0.4	0.4	0.4	0.5
Rhodococcus obuensis	33610	0.5	0.5	0.3	0.1
Rhodococcus sputi	29627	0.4	0.5	0.4	0.3

Example 9

Mycoplasmas are small, aerobic bacteria lacking cell walls. Mycoplasma pneumoniae is estimated to cause 8-15 million infections per year. The infections may be asymptomatic or range in severity from mild to severe bronchitis and pneumonia. The organism is believed to cause about 10% of pneumonias in the general population and 10-50% of the pneumonias of members of groups in prolonged, close contact such as college students and military personnel.

Diagnosis until now has required isolation of the organism in culture or demonstration of an increase in antibody titer. Culturing of the organism involves inoculation of respiratory tract specimens onto agar or biphasic media containing bacterial growth inhibitors. Examination for growth at 3-4 and 7-10 days is used to establish the presence or absence of any mycoplasma. Mycoplasma pneumoniae must then be identified by hemadsorption (the ability of M. pneumoniae to adhere sheep or guinea pig erythrocytes), hemolysis (the ability of M. pneumoniae to produce beta hemolysis of sheep or guinea pig erythrocytes in blood agar), growth inhibition by specific antibodies, or immunofluorescence with specific antibodies. The present invention has significant advantages over each of these prior art methods both because of the simplicity of the test and because of the greatly reduced time necessary to achieve a diagnosis.

A probe specific for the 5S rRNA of M. pneumoniae was obtained by a comparison of known rRNA sequences. The particular

sequences aligned were from M. pneumoniae, M. gallisepticum and
Ureaplasma urealyticum (Rogers, M.J. et al. 1985, Proc. Natl.
Acad. Sci. USA, 82 (1160-1164), M. capricolum (Hori, H. et al.
1981, Nucl. Acids Res. 9, 5407-5410) and Spiroplasma sp. (Walker,
5 R.T. et al. 1982 Nucl. Acids Res. 10, 6363-6367). The alignments
were performed as described above and outlined at page 6. 5S
rRNA can be isolated and sequenced as outlined in Rogers et al.,
or a primer can be made which is complementary to a conserved
region in the 5S rRNA and sequencing performed as outlined in
10 Examples 1-4. The conserved region of 5S rRNA is documented in
Fox, G.E. and Woese, C.R., 1975, Nature 256: 505-507. The
following sequence was determined to be specific for Mycoplasma
pneumoniae:

GCTTGGTGCTTTCCTATTCTCACTGAAACAGCTACATTCGGC.

15 The sequence is complementary to a unique segment found
in the 5S rRNA of Mycoplasma pneumoniae in the region
corresponding to bases 65-108 of E. coli 5S rRNA, and was
selected by comparison to 5S rRNA sequences from Mycoplasma
gallisepticum, Spiroplasma mirum and Ureaplasma urealyticum. The
20 oligonucleotide probe was characterized as described above. The
size of the probe was 42 bases. The probe has a T_m of 71.5°C.

To demonstrate the reactivity of this sequence for
Mycoplasma pneumoniae, the probe was tested in hybridization
reactions under the following conditions. ³²P-end-labelled
25 oligonucleotide probe was mixed with 1 microgram (7x10⁻¹³ moles)
of purified rRNA from Mycoplasma pneumoniae and reacted in 0.12 M
PB (equimolar amounts of Na₂HPO₄ and NaH₂PO₄), 1 mM EDTA and 0.2%
SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final
volume of 50 microliters. In separate tubes the probe was mixed

with the hybridization buffer with and without target Mycoplasma pneumoniae rRNA present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. These results are shown in Table 24.

5

TABLE 24

HYBRIDIZATION OF THE M. PNEUMONIAE 5S rRNA DNA
PROBE TO HOMOLOGOUS TARGET rRNA*

	<u>plus rRNA</u>	<u>minus rRNA</u>
10 <u>M. pneumoniae</u> 5S probe	85-95%	0.5%
* % Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$		

15 This data shows that the probe has a high extent of reaction to its homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the M. pneumoniae 5S probe was tested by mixing the ³²P labelled probe with rRNA released from cells from other Mycoplasma species. All hybridization assays were carried
20 out as described in Example 1. Table 25 indicates that the probe is specific for Mycoplasma pneumoniae and does not react with any other Mycoplasma species.

TABLE 25

25 HYBRIDIZATION OF M. PNEUMONIAE PROBE TO
OTHER MYCOPLASMA SPECIES

	<u>Acholeplasma laidlawii</u>	14089	3.3
	<u>M. buccale</u>	23636	1.7
	<u>M. capricolum</u>	23205	2.4
30	<u>M. columbinsale</u>	33549	1.4
	<u>M. faucium</u>	25293	1.4
	<u>M. fermentans</u>	15474	1.0
	<u>M. gallisepticum</u>	19610	1.8
	<u>M. gallopavonis</u>	33551	1.6

	M. genitalium	3353c	1.7
	M. hominis	14027	1.3
	M. orale	23714	1.8
	M. pneumoniae	15531	78.0
5	M. primatum	15497	1.6
	M. salivarium	23064	0.6
	Spiroplasma mirum		2.3

As shown in Table 26, the probe did not react with any other
10 closely related or phylogenetically diverse species of bacteria.

TABLE 26

HYBRIDIZATION OF M. PNEUMONIAE PROBE TO
A PHYLOGENETIC CROSS SECTION OF BACTERIA

15	Organism	ATCC#	% Probe Bound
	Corynebacterium xerosis	373	1.4
	Haemophilus influenzae	19418	1.4
	Klebsiella pneumoniae	23357	1.3
	Legionella pneumophila	33152	1.8
20	Mycobacterium tuberculosis (avir)	25177	1.6
	Mycoplasma pneumoniae	15531	52
	Neisseria meningitidis	13077	0.6
	Propionibacterium acnes	6919	2.0
	Pseudomonas aeruginosa	25330	1.6
25	Staphylococcus aureus	12598	2.0
	Streptococcus pneumoniae	c6306	1.9

Four additional probe sequences (numbered 2-5 below)
specific for Mycoplasma pneumoniae were obtained by utilizing
30 four unique primers complementary to conserved regions on 16S
rRNA. The regions correspond, respectively, to bases 190-230;
450-490; 820-860; and 1255-1290 of E. coli ^{16S} ~~16S~~ rRNA. Probe
B sequence #1 was obtained using a primer with the sequence 5'-
GGCCGTTACCCACCTACTAGCTAAT-3'. Probe sequence #2 was obtained
35 with a primer with the sequence 5'-GTATTACGCGGCTGCTGGC-3'.
Probe sequence #3 was obtained with a primer with the sequence
5'-CCGCTTGTGCGGGCCCCGTCAATTC-3'. Probe sequence #4 was obtained

using a primer with the sequence 5'-CGATTACTAGCGATTCC-3'. Sequencing reactions were performed as outlined in previous examples. The M. pneumoniae sequences were compared with sequences from Mycoplasma genitalium, Mycoplasma capricolum,
5 Mycoplasma gallisepticum and Spiroplasma mirum.

β The following probe sequences were characterized by criteria described in ^{Example 1} ~~example one~~ of the ^{patent} ~~parent~~ application and were shown to be specific for Mycoplasma pneumoniae:

2. AATAACGAACCCCTTGCAGGTCCTTTCAACTTTGAT
- 10 3. CAGTCAAACCTCTAGCCATTACCTGCTAAAGTCATT
4. TACCGAGGGGATCGCCCCGACAGCTAGTAT
5. CTTTACAGATTTGCTCACTTTTACAAGCTGGCGAC.

Probe #2 is 35 bases in length and has a T_m of 67°C. Probe #3 is 35 bases in length and has a T_m of 66°C. Probe #4 is 30 bases in
15 length and has a T_m of 69°C. Probe #5 is 35 bases long with a T_m of 66°C.

When the four probes were mixed and used in hybridization assays at 60°C in the same manner as previous examples, they were found to be specific for M. pneumoniae. The
20 probes do not cross react with other respiratory pathogens or with any organism representing the bacterial phylogenetic tree (Table 28).

TABLE 27
HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE
PROBES 2-5 TO MYCOPLASMA SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
5	Acholeplasma axanthum	27378	
	Acholeplasma laidlawii	14089	0.34
	Mycoplasma arginini	23838	0.30
	Mycoplasma arthritidis	19611	0.20
10	Mycoplasma bovigenitalium	19852	0.49
	Mycoplasma bovis	25523	0.18
	Mycoplasma buccale	23636	0.43
	Mycoplasma californicum	33451	0.37
	Mycoplasma capricolum	23205	0.79
15	Mycoplasma columbinasale	33549	0.38
	Mycoplasma columborale	29258	0.54
	Mycoplasma faucium	25293	0.50
	Mycoplasma fermentans	15474	0.45
	Mycoplasma gallisepticum	19610	0.27
20	Mycoplasma gallopavonis	33551	0.25
	Mycoplasma genitalium	33530	0.47
	Mycoplasma hominis	14027	2.5
	Mycoplasma hyorhinis	17981	0.52
	Mycoplasma orale	23714	0.46
25	Mycoplasma pneumoniae	15531	0.56
	Mycoplasma primatum	15497	34.0
	Mycoplasma pulmonis	19612	0.71
	Mycoplasma salivarium	23064	0.68
30	Spiroplasma citri	29416	0.46
	Spiroplasma mirum	29335	0.60
			0.52

TABLE 28
HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE
PROBES 2-5 WITH OTHER BACTERIA

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
35	Actinomyces israelii	10049	
	Bacteroides fragilis	23745	1.0
	Bifidobacterium breve	15700	1.4
	Bordetella bronchiseptica	10580	1.0
40	Clostridium innocuum	14501	0.9
	Clostridium pasteurianum	6013	1.0
	Clostridium perfringens	13124	0.9
	Clostridium ramosum	25582	1.1
	Corynebacterium xerosis	373	1.0
45	Erysipelothrix rhusiopathiae	19414	0.8
	Escherichia coli	11775	1.1
	Haemophilus influenzae	19418	1.0
	Klebsiella pneumoniae	15531	0.9
	Lactobacillus acidophilus	4356	1.0
50	Legionella pneumophila	33154	1.4
	Listeria monocytogenes	15313	0.8
			1.2

	Moraxella osloensis	19976	1.1
	Mycobacterium tuberculosis	25177	1.0
	Neisseria meningitidis	13077	1.0
	Pasteurella multocida	6529	1.6
5	Peptococcus magnus	14955	0.9
	Propionibacterium acnes	6919	1.1
	Pseudomonas aeruginosa	25330	1.0
	Staphylococcus aureus	12600	1.0
	Streptococcus faecalis	19433	1.5
10	Streptococcus mitis	9811	1.0
	Streptococcus pneumoniae	6306	1.0
	Streptococcus pyogenes	19615	1.1

Example 10

15 The genus Legionella contains 22 species which are all potentially pathogenic for humans. These organisms cause Legionnaires' disease, an acute pneumonia, or Pontiac fever, an acute, non-pneumonic, febrile illness that is not fatal.

Legionella species have also been shown to be
20 responsible for nosocomial pneumonia occurring predominantly among immunocompromised patients.

 Legionellosis, which includes Legionnaires' disease and Pontiac fever, is diagnosed on the basis of clinical symptoms, either direct or indirect fluorescence antibody tests, and by
25 culture using a buffered charcoal yeast extract (BCYE) agar containing selective antimicrobial agents. There is no single definitive genus test known in the prior art. (See Bergey's Manual of Systematic Bacteriology at page 283, (ed. 1984)). The fluorescent antibody tests are not able to identify all species
30 of Legionella, but only those few for which antibodies exist. The culture method is not definitively diagnostic for Legionella species.

 The oligonucleotide sequences described below, when used as probes in a nucleic acid hybridization assay, accurately
35 identify all species of Legionella. This assay is more sensitive

than culture or antibody tests and shortens significantly the time of identification and, thus, diagnosis. The assay, therefore, represents a significant improvement over prior diagnostic methods.

5 Three probe sequences specific for the genus Legionella were obtained by utilizing three unique primers complementary to conserved regions on both 16S and 23S rRNA. Sequence 1 was obtained by using a 16S primer with the sequence 5'-TCT ACG CAT TTC ACC GCT ACA C-3'. Probe sequence 2 was obtained with a 23S
10 primer of sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'. Probe sequence 3 was obtained with a 16S primer of sequence 5'-GCT CGT TGC GGG ACT TAA CCC ACC AT-3'. Sequencing with these primers was performed as described for previous examples.

15 The following three sequences were characterized by the criteria described in Example 1 and were shown to be specific for the genus Legionella. The phylogenetically nearest neighbors Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus and Acinetobacter calcoaceticus were used as comparisons with sequences from Legionella species.

- 20 1. TACCCTCTCCCATACTCGAGTCAACCAGTATTATCTGACC
 2. GGATTTCACGTGTCCCGGCCTACTTGTTCTGGGTGCGTAGTTC
 3. CATCTCTGCAAAATTCACCTGTATGTCAAGGGTAGGTAAGG.

Sequence 1, from 16S rRNA, is 40 bases in length and has a T_m of 72°C. Sequence 2, from 23S rRNA, is 42 bases in length and has a
25 T_m of 73°C. Sequence 3, from 16S rRNA, is 40 bases in length and has a T_m of 68°C. These sequences are capable of hybridizing to RNA of the genus Legionella in the regions corresponding respectively to, 630-675 of E. coli ^{16S}~~16S~~ rRNA; 350-395 of E. coli ^{23S}~~23S~~ rRNA; and 975-1020 of E. coli ^{16S}~~16S~~ rRNA. When mixed together

the probes had a combined average T_m of 73°C. Analysis on polyacrylamide gels showed that each probe was the correct length and sequence analysis demonstrated that each was the correct sequence of bases.

5 When the three probes were mixed and used in a hybridization assay, they were found to be specific for the genus Legionella (Tables 29 and 30) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 31 and 32). Use of more than one
10 probe, i.e., a mixture of probes, can result in increased assay sensitivity and/or in an increase in the number of non-viral organisms to be detected.

TABLE 29

HYBRIDIZATION OF LEGIONELLA
PROBES TO HOMOLOGOUS TARGET rRNA

	plus rRNA	minus rRNA
<u>Legionella</u> probe	80%	1.0%

TABLE 30

HYBRIDIZATION OF LEGIONELLA
PROBES TO LEGIONELLA SPECIES

	Organism	ATCC#	% Probes Bound
	L. anisa	35292	42.0
25	L. bozemanii	33217	58.0
	L. cherrii	35252	69.0
	L. dumoffii	33279	57.0
	L. erythra	CDC#9PlW044C	26.0
	L. feeleii	35303	59.0
30	L. hackeliae	35250	47.0
	L. jamestowniensis	35298	20.0
	L. jordanis	33623	50.6
	L. longbeachae	33484	48.0
	L. maceachernii	35300	25.0
35	L. micdadei	33704	38.0
	L. oakridgensis	33761	44.0

	L. parisiensis	9060	69.0
	L. pneumophila 1*	6736	75.0
	" 2		64.0
5	" 3		73.0
	" 4		73.0
	" 5		78.0
	" 6		75.0
	" 7		73.0
10	" 8		63.0
	" 11		75.0
	L. rubrilucens	35304	12.0
	L. sainthelensi	35248	61.0
	L. sainticrucis	35301	24.0
	L. spiritensis	CDC#MSH9	55.0
15	L. steigerwaltii	7430	56.0
	L. wadsworthii	33877	37.0

* The numbers 1-8 and 11 are serotypes of L. pneumophila.

TABLE 31

HYBRIDIZATION OF LEGIONELLA PROBES TO
RESPIRATORY PATHOGENS

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Corynebacterium xerosis	373	2.1
	Haemophilus influenzae	19418	2.3
25	Klebsiella pneumoniae	23357	2.0
	Mycoplasma pneumoniae	15531	2.3
	Neisseria meningitidis	13090	2.2
	Pseudomonas aeruginosa	25330	1.2
	Propionibacterium acnes	6919	1.6
30	Streptococcus pneumoniae	6306	0.8
	Staphylococcus aureus	25923	1.6

TABLE 32

HYBRIDIZATION OF LEGIONELLA PROBES TO
A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	1.4
	Branhamella catarrhalis	25238	2.0
	Bacillus subtilis	6051	1.9
40	Bacteroides fragilis	23745	2.2
	Campylobacter jejuni	33560	1.2
	Chromobacterium violaceum	29094	1.3
	Clostridium perfringens	13124	1.9
	Deinococcus radiodurans	35073	1.8
45	Derxia gummosa	15994	2.0
	Enterobacter aerogenes	13048	1.4

	Escherichia coli	11775	1.2
	Mycoplasma hominis	14027	1.1
	Proteus mirabilis	29906	1.4
5	Pseudomonas cepacia	11762	1.1
	Rahnella aquatilis	33071	1.7
	Rhodospirillum rubrum	11170	2.0
	Streptococcus mitis	9811	2.0
	Vibrio parahaemolyticus	17802	2.0
10	Yersinia enterocolitica	9610	1.2

Three additional probe sequences (numbered 4-6) specific for the genus Legionella were obtained by utilizing two primers complementary to conserved regions on 23S rRNA. Sequence 4 was made from a 23S primer with the sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Probe sequences 5 and 6 were made from a 23S primer of sequence 5'-AAG CCG GTT ATC CCC GGG GTA ACT TTT-3". Sequencing with these primers was performed as described for previous examples.

The following three sequences were characterized by the criteria previously described and were shown to be specific for the genus Legionella. The phylogenetically nearest neighbors Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus and Actinobacter calcoaceticus were used for comparisons with sequences from Legionella species.

- 25 4. GCG GTA CCG TTC TCT ATA AGT TAT GGC TAG C
5. GTA CCG AGG GTA CCT TTG TGC T
6. CAC TCT TGG TAC GAT GTC CGA C

Probe 4, complementary to 23S rRNA in the region corresponding to bases 1585-1620 of E. coli ^{23S} rRNA, is 31 bases long and has a Tm of 67°C. Probe 5, complementary to 23S rRNA in the region corresponding to bases 2280-2330 of E. coli ^{23S} rRNA, is 22 bases long and has a Tm of 66°C. Probe 6, complementary to

23S rRNA in the same region as Probe 5, is 22 bases long and has a Tm of 63°C.

When the three probes were mixed with probe 3 above and used in a hybridization assay as described for probes 1-3, they were found to be specific for the genus Legionella (Table 33) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 34 and 35). Using more than one probe, i.e., a mixture of probes, can improve assay sensitivity and/or increase the number of non-viral organisms detected.

TABLE 33
HYBRIDIZATION OF LEGIONELLA PROBES TO
LEGIONELLA SPECIES

	Organism	ATCC#	% Probes Bound
15	L. anisa	35292	29.6
	L. bozemanii	33217	35.5
	L. cherrii	35252	29.2
	L. dumoffii	33279	26.0
20	L. erythra	35303	32.0
	L. feelii	CDC#9P1WO44C	32.0
	L. hackeliae	35250	39.0
	L. jamestowniensis	35298	31.2
	L. jordanis	33623	25.7
25	L. longbeachae	33484	27.6
	L. maceahernii	35300	39.3
	L. micdadei	33204	31.0
	L. oakridgensis	33761	24.4
	L. parisiensi	35299	31.2
30	L. pneumophila 1*	33153	40.0
	" 2	33154	38.5
	" 3	33155	44.6
	" 4	33156	48.6
	" 5	33216	32.0
35	" 6	33215	43.0
	" 7	33823	29.5
	" 8	35096	37.6
	" 11	43130	44.5
	L. rubrilucens	35304	30.1
40	L. sainthelensis	35248	27.0
	L. sainticrucis	35301	22.0

L. spiritensis	CDC#MSH9	40.5
L. steigerwaltii	35302	31.7
L. wadsworthii	33877	30.0

* The numbers 1-8 and 11 are serotypes of *L. pneumophila*.

5

TABLE 34

HYBRIDIZATION OF LEGIONELLA PROBES TO
RESPIRATORY PATHOGENS

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Corynebacterium xerosis	373	0.13
	Haemophilum influenzae	19418	0.12
	Klebsiella pneumoniae	23357	0.13
	Neisseria meningitidis	13090	0.14
	Pseudomonas aeruginosa	25330	0.13
15	Propionibacterium acnes	6919	0.11
	Streptococcus pneumoniae	6306	0.08
	Staphylococcus aureus	25923	0.15

TABLE 35

HYBRIDIZATION OF LEGIONELLA PROBES TO
A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	0.12
	Branhamella catarrhalis	25238	0.13
25	Bacillus subtilis	6051	0.09
	Bacteroides fragilis	23745	0.12
	Campylobacter jejuni	33560	0.06
	Chromobacterium violaceum	29094	0.33
	Clostridium perfringens	13124	0.07
30	Deinococcus radiodurans	35073	0.11
	Derrxia gummosa	15994	0.15
	Enterobacter aerogenes	13048	0.26
	Escherichia coli	11775	0.09
	Mycoplasma hominis	14027	0.09
35	Proteus mirabilis	29906	0.09
	Pseudomonas cepacia	17762	0.20
	Rahnella aquatilis	33071	0.15
	Rhodospirillum rubrum	11170	0.13
	Streptococcus mitis	9811	0.07
40	Vibrio parahaemolyticus	17802	0.11
	Yersinia enterocolitica	9610	0.19

Example 11

Chlamydia are gram-negative, non-motile, obligate intracellular bacteria. The species C. trachomatis is associated with endemic trachoma (the most common preventable form of blindness), inclusion conjunctivitis and lymphogranuloma venereum (LGV). It is a major cause of nongonococcal urethritis in men and may cause cervicitis and acute salpingitis in women. Eye disease or chlamydial pneumonia may develop in newborns passing through the infected birth canal.

There are several methods known in the art for identification of C. trachomatis in the urogenital tract, for example, by direct immunofluorescent staining or enzyme immunoassay of clinical specimens. The method of choice, however, remains culture of the organism in cycloheximide treated McCoy cells. Cell culture is followed by morphological or fluorescent antibody staining for confirmation of the organism's identity.

The inventive oligonucleotide sequences described below, when used as probes in nucleic acid hybridization assay, accurately identify Chlamydia trachomatis isolates. This assay test is equal in sensitivity to culture or antibody tests and, in the case of culture, significantly shortens the time to identification, and thus, diagnosis.

The use of probes to identify and distinguish between members of the species is novel and inventive. Indeed, Kingsbury, D.T., and E. Weiss, 1968 J. Bacteriol. 96: 1421-23 (1968); Moulder, J.W., ASM News, Vol.50, No.8, (1984) report a 10% DNA homology between C. trachomatis and C. psittaci. Moreover, these reports show that different C. trachomatis

strains differ in DNA homology. Weisberg, W.G. et. al, J. Bacteriol. 167:570-574 (1986) published the 16S rRNA sequences of C. psittaci and noted that C. trachomatis and C. psittaci share a greater than 95% rRNA homology. From these reports, it may be
5 inferred that it would be difficult to invent (1) probes capable of hybridizing to all strains of C. trachomatis; and (2) probes capable of distinguishing between C. trachomatis and C. psittaci. The following probes accomplish both objectives.

Ten probe sequences specific for Chlamydia trachomatis
10 were made using seven unique primers complementary to conserved regions of both 16S and 23S rRNA. Probe sequence 1 was obtained from a 16S primer of sequence 5'-TCT ACG CAT TTC ACC GCT ACA C-3'. Probe sequence 2 was obtained with a 16S primer of sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. Sequences 3 and 4 were
15 obtained using a 16S primer with the sequence 5'-GGC CGT TAC CCC ACC TAC TAG CTA AT-3'. Probe sequences 5 and 6 were obtained with a 23S primer of sequence 5'-CTT TCC CTC ACG GTA-3'. Probe sequences 7 and 8 were obtained with a 23S primer of sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Probe sequence 9 was obtained with
20 a 23S primer of sequence 5'-TCG GAA CTT ACC CGA CAA GGA ATT TC-3'. Probe sequence 10 was obtained with a primer of sequence 5'-CTA CTT TCC TGC GTC A-3'.

The following ten sequences were characterized using the criteria described in Example 1 and were shown to be specific
25 for the rRNA of Chlamydia trachomatis. The phylogenetically nearest neighbor Chlamydia psittaci was used for comparison with Chlamydia trachomatis sequence.

1. CCG ACT CGG GGT TGA GCC CAT CTT TGA CAA
2. TTA CGT CCG ACA CGG ATG GGG TTG AGA CCA TC

3. CCG CCA CTA AAC AAT CGT CGA AAC AAT TGC TCC GTT
CGA
4. CGT TAC TCG GAT GCC CAA ATA TCG CCA CAT TCG
5. CAT CCA TCT TTC CAG ATG TGT TCA ACT AGG AGT CCT
GAT CC
6. GAG GTC GGT CTT TCT CTC CTT TCG TCT ACG
7. CCG TTC TCA TCG CTC TAC GGA CTC TTC CAA TCG
8. CGA AGA TTC CCC TTG ATC GCG ACC TGA TCT
9. CCG GGG CTC CTA TCG TTC CAT AGT CAC CCT AAA AG
10. TAC CGC GTG TCT TAT CGA CAC ACC CGC G

Sequence 1, from 16S rRNA, is 30 bases in length and has a T_m of 66°C. Sequence 2, from 16S rRNA, is 32 bases in length and has a T_m of 67°C. Sequence 3, from 16S rRNA, is 39 bases in length and has a T_m of 70°C. Sequence 4, from 16S rRNA, is 33 bases in length and has a T_m of 69°C. Sequence 5, from 23S rRNA, is 41 bases in length and has a T_m of 71°C. Sequence 6, from 23S rRNA, is 30 bases in length and has a T_m of 72°C. Sequence 7, from 23S rRNA, is 33 bases in length and has a T_m of 72°C. Sequence 8, from 23S rRNA, is 30 bases in length and has a T_m of 71°C. Sequence 9, from 23S rRNA is 35 bases in length and has a T_m of 74°C. Sequence 10 is 28 bases in length and has a T_m of 72°C.

The reactivity and specificity of the probes was tested by hybridization assays. ³²P-end-labeled oligonucleotide probes 1 and 2 were mixed with purified RNA or RNA released from at least 10⁷ organisms in 0.55 ml of 41% diisobutyl sulfosuccinate, 3% sodium dodecyl sulfate, 0.03 M sodium phosphate pH 6.8, 1 mM EDTA, 1 mM EGTA at 60°C (probe 1) or 64°C (probe 2) for 1 hour. Hybrids were bound to hydroxyapatite as described in previous examples

and the amount of radioactivity bound was determined by scintillation counting. Table 36 shows that probes 1 and 2 hybridize well to all serotypes of C. trachomatis tested. Probe 1 does not react with any strain of C. psittaci tested and probe 2 does not react with two of the strains. Probe 2 does react with the ovine polyarthrititis strain of C. psittaci, an organism which is not known to infect humans. Table 37 demonstrates the reactivity and specificity of probes 3-9 when ¹²⁵I-labeled and used as a mix. In this case, the hybrids were bound to cationic magnetic particles as described in Arnold et al., U.S. Patent App. Ser. No. 020,866 filed March 2, 1987. These probes hybridize well to all strains of C. trachomatis tested and not to any strains of C. psittaci. Probes 3-9 were further tested against a panel of organisms commonly found in the urogenital tract (Table 38) and a phylogenetic cross section of organisms (Table 39). In all cases, the probes were shown to be specific. Probe 10 is 25% non-homologous to C. psittaci and also should be specific for C. trachomatis.

TABLE 36

HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 1 AND 2
TO CHLAMYDIA RNA

	Organism	ATCC#	% Probe Bound	
			Probe 1	Probe 2
25	Chlamydia trachomatis serotype C	VR578	22	39
	Chlamydia trachomatis serotype E	VR348B	27	48
	Chlamydia trachomatis serotype G	VR878	20	44
	Chlamydia trachomatis serotype I	VR880	20	42
	Chlamydia trachomatis serotype K	VR887	28	45
30	Chlamydia psittaci guinea pig conjunctivitis strain	VR813	1.2	1.4
	Chlamydia psittaci ovine abortion strain	VR656	1.0	3.0
35	Chlamydia psittaci ovine polyarthrititis strain	VR619	1.1	35.3

TABLE 37
HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9
WITH CHLAMYDIA rRNA

	Organism	Serovar	ATCC#	Ratio Counts Bound*
5	C. trachomatis	A		689
	C. trachomatis	B		560
	C. trachomatis	Ba		1066
10	C. trachomatis	C	VR548	962
	C. trachomatis	D		1192
	C. trachomatis	E	VR348	1022
	C. trachomatis	F		391
	C. trachomatis	G	VR878	874
15	C. trachomatis	H		954
	C. trachomatis	I	VR880	943
	C. trachomatis	J		482
	C. trachomatis	K	VR887	999
	C. trachomatis	L1		638
20	C. trachomatis	L2		501
	C. trachomatis	L3	VR903	821
	C. psittaci		VR125	1.6
	C. psittaci		VR629	0.9
	C. psittaci		VR656	1.3
25	C. psittaci		VR813	1.2

*Ratio = $\frac{\text{counts bound when RNA present}}{\text{counts bound when no RNA present}}$

TABLE 38
HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9
TO ORGANISMS FOUND IN THE UROGENITAL TRACT/

	Organism	ATCC#	Ratio Counts Bound*
35	Achromobacter xylosoxidans	27061	1.9
	Acinetobacter lwoffii	15309	1.2
	Branhamella catarrhalis	25238	1.2
	Candida albicans	18804	2.4
	Flavobacterium meningosepticum	13253	1.1
	Gardnerella vaginalis	14018	1.3
40	Lactobacillus acidophilus	4356	0.8
	Listeria monocytogenes	15313	0.7
	Mycobacterium smegmatis	14468	1.1
	Moraxella osloensis	19976	1.3
	Neisseria gonorrhoeae	19424	2.3
45	Pasteurella multocida	6529	1.0
	Peptostreptococcus anaerobius	27337	1.2
	Streptococcus agalactiae	13813	4.0
	Streptococcus faecalis	19433	2.6

*Ratio = $\frac{\text{counts bound when RNA present}}{\text{counts bound when no RNA present}}$

TABLE 39

HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9
TO PHYLOGENETICALLY DIVERSE ORGANISMS/

Organism	ATCC#	Ratio Counts Bound*
Bacillus subtilis	6051	2.2
Bacteroides fragilis	23745	1.6
Campylobacter jejuni	33560	1.4
Chromobacterium violaceum	29094	1.4
Deinococcus radiodurans	35073	1.8
Derxia gummosa	15994	1.3
Enterobacter aerogenes	13048	1.9
Escherichia coli	11775	1.9
Mycoplasma hominis	14027	1.3
Pseudomonas cepacia	17762	2.2
Proteus mirabilis	29906	2.2
Rahnella aquatilis	33071	1.9
Rhodospirillum rubrum	11170	1.9
Vibrio parahaemolyticus	17802	2.0
Yersinia enterocolitica	9610	2.5

*Ratio = $\frac{\text{counts bound when RNA present}}{\text{counts bound when no RNA present}}$

Example 12

Campylobacters are motile, microaerophilic, gram negative curved rods. The genus is quite diverse and distinct from other genera. Although the genus is well defined, some revision is occurring at the species level (Romaniuk, P.J. et al., J. Bacteriol. 169:2137-2141 (1987)). Three Campylobacter species, Campylobacter jejuni, C. coli and C. lariidis, cause enteritis in humans. The disease includes diarrhea, fever, nausea, abdominal pain and in some cases, vomiting. These organisms cause an estimated 2 million infections per year in the United States (estimate based on the number of Salmonella and Shigella induced cases of diarrheal disease). Other members of

the genus cause septicemias in humans and abortion and infertility in sheep and cattle.

Diagnosis of Campylobacter enteritis is currently dependent upon growth and isolation of the organism in culture, followed by a number of biochemical tests. Optimum growth of campylobacters requires special conditions such as low oxygen tension and high temperature (42°C). No single set of conditions is recommended for isolation of all Campylobacter species.

The oligonucleotide sequences listed below, when used in a hybridization assay, hybridize to the 16S rRNA of the Campylobacter species of interest. The present invention has significant advantages over the prior art methods of detection of Campylobacter because one probe can detect all Campylobacters of interest; the other two probes detect the enteric Campylobacters and one can detect human isolates of Campylobacter. In addition, the probes have advantages over the prior art in terms of ease of the assay and greatly reduced time to identification and therefore, diagnosis.

The four probes which hybridize to the 16S rRNA of Campylobacter species of interest were constructed using three unique primers complementary to 16S rRNA. Sequences 1 and 2 were made using a 16S primer with the sequences 5'-GTA TTA CCG CGG CTG CTG GCA C-3'. Sequence 3 was made using a 16S primer with the sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. Sequence 4 was made with a 16S primer with the sequence 5'-GCT CGT TGC GGG ACT TAA CCC AAC AT-3'.

The following sequences were characterized and shown to hybridize to Campylobacter jejuni, C. coli and C. lariidis. The phylogenetically nearest neighbors Vibrio parahaemolyticus and

Wollinella succinogenes were used for comparison with the campylobacter sequences.

1. CGC TCC GAA AAG TGT CAT CCT CC
2. CCT TAG GTA CCG TCA GAA TTC TTC CC
3. GCC TTC GCA ATG GGT ATT CTT GGT G
4. GGT TCT TAG GAT ATC AAG CCC AGG

Sequence 1, from 16S rRNA, is 23 bases in length and has a Tm of 65°C. Sequence 2, from 16S rRNA, is 26 bases in length and has a Tm of 64°C. Sequence 3, from 16S rRNA, is 25 bases in length and has a Tm of 66°C. Sequence 4, from 16S rRNA, is 24 bases in length and has a Tm of 61°C. Sequence 1 is capable of hybridizing in the region corresponding to bases 405-428 of E. coli ^{16S}~~16S~~ rRNA; Sequence 2 is capable of hybridizing in the region corresponding to bases 440-475 of E. coli ^{16S}~~16S~~ rRNA; Sequence 3 is capable of hybridizing in the region corresponding to bases 705-735 of E. coli ^{16S}~~16S~~ rRNA; Sequence 4 is capable of hybridizing in the region corresponding to bases 980-1010 of E. coli ^{16S}~~16S~~ rRNA.

The reactivity and specificity of the probes for campylobacter was tested in hybridization assays. ³²P-end-labeled oligonucleotide probes were mixed with purified RNA or RNA released from cells in 0.1% sodium dodecyl sulfate. 0.5 ml of hybridization solution (41% diisobutyl sulfosuccinate, ^{30 mM}~~30 mM~~ sodium phosphate, pH 6.8, 0.7% sodium dodecyl sulfate, ^{1 mM}~~1 mM~~ EDTA, ^{1 mM}~~1 mM~~ EGTA) was added and the mixture incubated at 60°C for 1 to 1.5 hour. Following incubation, 2 to 2.5 ml of separation solution (2% hydroxyapatite, 0.12 M sodium phosphate, ^{pH 6.8}~~pH 6.8~~, 0.02% sodium dodecyl sulfate) was added and the mixture incubated at 60°C for five minutes. The sample was centrifuged and the

supernatant removed. 2.5 ml of wash solution (0.12 M sodium phosphate, ^{pH 6.8} ~~pH 6.0~~, 0.02% sodium dodecyl sulfate) was added and the sample mixed, centrifuged and the supernatant removed. The radioactivity bound to the hydroxyapatite was determined by scintillation counting.

Table 40 indicates that the probes hybridize well to the Campylobacter species of interest, C. jejuni, C. coli, and C. laridis. Probe 1 detects all of the Campylobacter species tested, probes 2 and 4 detect only the enteric campylobacters, and probe 3 detects all of the Campylobacter species except C. sputorum, an organism isolated from cattle. Thus all of the probes are useful for identifying campylobacter in stool samples. The choice of which probe to use for other applications would depend upon the level of specificity required (i.e., enteric campylobacters, or all Campylobacter species).

TABLE 40

HYBRIDIZATION OF CAMPYLOBACTER PROBES 1-4
TO CAMPYLOBACTER SPECIES/

Organism	ATCC#	% Probe Bound (*)			
		1	2	3	4
Campylobacter coli	33559	64	70	52	49
C. fetus					
subsp. fetus	27374	68	0.1	66	0.5
C. fetus					
subsp. venerealis	19438	66	0.7	54	1.2
C. jejuni	33560	63	76	51	56
C. laridis	35221	74	73	64	52
C. sputorum					
subsp. bubulus	33562	71	3.0	2.5	0

(*) % Probe Bound = cpm bound to hydroxyapatite-cpm bound when no RNA present/total cpm used in the assay

Table 41 shows that the probes do not hybridize to closely related organisms or organisms found in the gastrointestinal tract.

TABLE 41

HYBRIDIZATION OF CAMPYLOBACTER PROBES 1-4 TO CLOSELY RELATED ORGANISMS AND ORGANISMS FOUND IN THE GASTRO-INTESTINAL TRACT/

Organism	ATCC#	% Probe Bound (*)			
		1	2	3	4
Bacteroides fragilis	25285	0	0.2	0.7	0
Escherichia coli	11775	1.3	0.5	0.5	0
Salmonella typhimurium	14028	0	0	0.3	0
Shigella boydii	29929	0	0.2	0.5	0
Shigella dysenteriae	13313	0	0.7	0.2	0
Shigella flexneri	29903	0	0	0.5	0
Shigella sonnei	29930	0	0	0.1	0
Vibrio parahaemolyticus	17802	0	1.9	0.1	0
Wollinella succinogenes	29543	0.4	2.1	2.2	0
Yersinia pseudotuberculosis	29833	0.6	0.2	1.7	0.3

(*) % probe bound = cpm bound to hydroxyapatite-cpm bound when no RNA present/total cpm used in the assay

The probes specific for the enteric Campylobacters, probes 2 and 4, were further tested and shown not to react with rRNAs of other organisms found in the gastrointestinal tract.

TABLE 42

HYBRIDIZATION OF CAMPYLOBACTER PROBES 2 AND 4 TO ORGANISMS FOUND IN THE GASTROINTESTINAL TRACT/

Organism	ATCC#	% Probe Bound (*)	
		Probe 2	Probe 4
Citrobacter diversus	27156	0	0
Clostridium perfringens	13124	0	0
Enterobacter cloacae	13047	0	0
Klebsiella pneumoniae	23357	0	0.5
Proteus mirabilis	25933	0	0
Serratia marcescens	13880	0	0
Staphylococcus aureus	412600	0	0
Staphylococcus epidermidis	14990	0	0.3
Streptococcus bovis	33317	0	0

(*) % probe bound = cpm bound to hydroxyapatite-cpm bound when no RNA present/total cpm used in the assay

Example 13

5 Streptococci are gram positive, oxidase negative
coccoid bacteria. The genus has been divided into 18 groups, A-
R, on the basis of group-specific carbohydrates. Group D
streptococci are further subdivided into the enterococci (S.
10 faecium, S. faecalis, S. avium and S. gallinarum and the non-
enterococci S. bovis and S. equinus. S. faecium, S. faecalis and
S. avium are considered the medically important enterococci.
Some species of streptococcus are human pathogens; others are
normal flora in the mouth and intestine but are capable of
causing disease when introduced to other sites. Two examples are
15 S. faecium and S. faecalis which are normally found in the
intestine but may spread to cause bacteremia, wound infections,
and as many as 10% of the urinary tract infections in the United
States.

20 Current methods of detection of enterococci require
culture of the specimen for 18-72 hours followed by a battery of
biochemical tests. The oligonucleotide sequence shown below,
when used in a hybridization assay, accurately detects
Streptococcus faecalis, S. avium, and S. faecium. The inventive
probe does not cross react with other Streptococci or
25 Staphylococci which are very closely related in DNA homology.
(Kiepper-Baez, 1981, 1982, Schliefer 1984.) The current
invention also reduces the number of tests which must be run on a
sample and greatly reduces the time to identification and thus,
diagnosis. This represents a significant improvement over prior
30 art methods.

The probe sequence was identified using a primer complementary to 16S rRNA with the sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. The following sequence was characterized and shown to be specific for three enterococci, S. faecium, S.

5 faecalis and S. avium. The phylogenetically nearest neighbors S. agalactiae, S. bovis, S. pneumoniae and S. pyogenes were used for comparison with the sequences of interest.

1. TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA

B The sequence is 35 bases in length and has a T_m of 10 72°C. It is capable of hybridizing in the region corresponding to bases 825-860 of E. coli ^{16S} ~~16S~~ rRNA. To demonstrate the reactivity and specificity of the probe, it was used in a hybridization assay with purified RNA or RNA released from cells. A suspension containing at least 10⁷ cells in 2% sodium dodecyl 15 sulfate was vortexed in the presence of glass beads. 0.1 ml of suspension was mixed with 0.1 ml of hybridization buffer (0.96 M sodium phosphate, pH 6.8, 0.002 M EDTA, 0.002 M EGTA) and incubated at 65°C for 2 hours. After incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium 20 dodecyl sulfate was added and the mixture was incubated at 65°C for 10 minutes. The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M phosphate buffer, pH 6.8, 0.02% sodium dodecyl sulfate) was added and the samples were vortexed, centrifuged, and the supernatant removed. The amount 25 of radioactivity bound to the hydroxyapatite was determined by scintillation counting. Table 43 shows that the probe reacts well with S. faecium, S. faecalis, and S. avium, and does not react with other closely related organisms.

TABLE 43
HYBRIDIZATION OF THE ENTEROCOCCUS PROBE
TO CLOSELY RELATED ORGANISMS/

Organism	ATCC#	% Probe Bound
Staphylococcus aureus	12600	1.4
Streptococcus agalactiae	13813	1.5
Streptococcus avium	14025	22.7
Streptococcus bovis	33317	1.4
Streptococcus faecalis	19433	45.3
Streptococcus faecium	19434	43.0
Streptococcus mitis	9811	1.5
Streptococcus pneumoniae	6306	1.5
Streptococcus pyogenes	19615	1.3

Example 14

Pseudomonads are gram-negative, nonsporeforming, nonfermentative bacilli. Pseudomonads are common inhabitants of soil and water and rarely infect healthy individuals. When the organisms encounter already compromised patients, they can cause a variety of clinical syndromes including wound infections, post-surgical infections, septicemia, infant diarrhea and respiratory and urinary tract infections. Members of the genus Pseudomonas are particularly important to identify in a clinical sample because of the resistance of the organisms to antibiotics. Nucleic acid homology studies have divided the genus into five homology classes known as RNA groups I-V. Eighty-three percent of all clinical isolates of Pseudomonas are from RNA group I and Pseudomonas aeruginosa is by far the most common species isolated.

Current methods of detection of pseudomonas require culture of a patient sample for 24-72 hours, followed by a battery of biochemical tests. The oligonucleotide sequence below, when used in a hybridization assay, detects the clinically

important group I pseudomonas. The present invention reduces the number of tests which must be run on a sample, and reduces the time to detection. This represents a significant improvement over prior art methods.

5 The sequence was obtained with a primer complementary to a conserved region on 23S rRNA with the sequence 5'-CTT TCC CTC ACG GTA-3'. The following sequence was shown to detect group I pseudomonads:

1. CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT

10 The probe is 35 bases in length and has a T_m of 70°C. It is capable of hybridizing to the RNA of group I Pseudomonas in the region corresponding to bases 365-405 of E. coli ^{23S} ~~23S~~ rRNA. To demonstrate the reactivity and specificity of the probe, it was used in a hybridization assay. ³²P-end-labeled oligonucleo-
15 tide was mixed with RNA released from at least 10⁷ organisms by standard methods in 0.48 M sodium phosphate pH 6.8, 1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA and incubated at 65°C for two hours. After incubation, the RNA:DNA hybrids were bound to hydroxyapatite as described for previous examples and the radio-
20 activity bound was determined by scintillation counting. Table 44 demonstrates that the probe reacted well with all 8 species of group I pseudomonads that were tested. The probe did not react with RNA from group II or group V organisms. A low reaction was seen with Pseudomonas acidovorans, a group III organism which re-
25 presents < 1% of all isolates of nonfermentative bacilli from clinical samples. Table 45 demonstrates that the probe does not react with other closely related organisms which were tested.

TABLE 44

HYBRIDIZATION OF PSEUDOMONAS GROUP I
PROBE TO PSEUDOMONAS RNAs

Organism	Group	ATCC#	% Probe* Bound
Pseudomonas alcaligenes	I	14909	24
Pseudomonas aeruginosa	I	10145	83
Pseudomonas denitrificans	I	13867	83
Pseudomonas fluorescens	I	13525	82
Pseudomonas mendocina	I	25411	79
Pseudomonas pseudoalcaligenes	I	17440	78
Pseudomonas putida	I	12633	80
Pseudomonas stutzeri	I	17588	84
Pseudomonas cepacia	II	25416	0
Pseudomonas pickettii	II	27511	1.0
Pseudomonas acidovorans	III	15668	11
Pseudomonas maltophilia	V	13637	0.2

*% Probe Bound = counts bound when RNA present - counts bound when no RNA present/total counts used in the assay

TABLE 45

HYBRIDIZATION OF PSEUDOMONAS GROUP I
PROBE TO RNAs OF CLOSELY RELATED ORGANISMS

Organism	ATCC#	% Probe* Bound
Acinetobacter calcoaceticus	23055	1.6
Legionella pneumophila	33155	0.6
Moraxella phenylpyruvica	23333	0.3
Morganella morganii	25830	0
Vibrio parahaemolyticus	17802	0.6

*% Probe Bound = counts bound when RNA present - counts bound when no RNA present/total counts used in the assay

Example 15

Examples 15-18 disclose probes for the Enterobacteriaceae, all of which are highly related at the DNA level. Even fewer differences exist at the rRNA level. For example, Proteus vulgaris ^{16S} rRNA is 93% homologous to E. coli. These factors illustrate the difficulties associated with making rRNA

probes specific for this group of organisms. Nevertheless, we have invented probes for Enterobacter cloacae, Proteus mirabilis, Salmonella and E. coli.

Members of the genus Enterobacter are motile, gram
5 negative, non-sporeforming bacilli which belong in the family Enterobacteriaceae. The genus is a large and heterogeneous group. Eight species have been defined but only 5 are clinically significant. Enterobacter cloacae and E. aerogenes are the most common isolates and are associated with genitourinary, pulmonary,
10 blood, central nervous system and soft tissue infections in humans.

The current method for identifying Enterobacter cloacae from patient samples involves culture of the specimen on agar plates for 18-24 hours, followed by a battery of biochemical
15 tests. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately identifies Enterobacter cloacae. The present invention reduces the number of tests which must be run on a sample, the time to identification and therefore, diagnosis, and thus represents a
20 significant improvement over prior art methods.

The probe specific for Enterobacter cloacae was obtained with a primer complementary to a conserved region of 23S rRNA with the sequence 5'-CAG TCA GGA GTA TTT AGC CTT-'3.

The following sequence was characterized and shown to
25 be specific for E. cloacae. The phylogenetically nearest neighbors Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella enteritidis, and Citrobacter freundii were used as comparisons with the sequence of E. cloacae.

1. GTG TGT TTT CGT GTA CGG GAC TTT CAC CC

The probe is 29 bases in length and has a T_m of 68°C. It is capable of hybridizing to RNA of *E. cloacae* in the region corresponding to bases 305-340 of *E. coli* ⁸³⁵ ~~235~~ rRNA. To demonstrate the reactivity and specificity of the probe for *E. cloacae*, it was used in a hybridization assay. ³²P-end-labeled oligonucleotide probe was mixed with RNA released from at least 10^7 organisms in 1% sodium dodecyl sulfate, 0.48 M sodium phosphate, pH 6.8 (0.2 ml final volume) and incubated at 60°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 60°C for 10 minutes. The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 46 and demonstrates that the probe reacts well with *E. cloacae* and does not react with the RNA of closely related organisms.

TABLE 46

HYBRIDIZATION OF ENTEROBACTER CLOACAE PROBE
TO CLOSELY RELATED ORGANISMS

Organisms Name	ATCC#	% Probe Bound
Citrobacter freundii	8090	1.8
Enterobacter aerogenes	13048	1.4
Enterobacter cloacae	13047	27.
Escherichia coli	11775	1.0
Klebsiella pneumoniae	13883	1.7
Proteus mirabilis	29906	0.9
Proteus vulgaris	13315	0.6
Providencia stuartii	29914	1.1

Table 47 shows that the probe does not react with the RNA of organisms found in urine.

TABLE 47

HYBRIDIZATION OF ENTEROBACTER CLOACAE
PROBE TO ORGANISMS FOUND IN URINE/

	<u>Organisms Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Candida albicans	18804	0.8
	Candida krusei	34135	0.8
	Candida parapsilosis	22019	0.9
	Candida tropicalis	750	1.1
	Pseudomonas aeruginosa	10145	1.0
15	Serratia marcescens	13880	1.6
	Staphylococcus aureus	12600	1.7
	Staphylococcus epidermidis	14990	1.4
	Streptococcus agalactiae	13813	2.5
	Streptococcus faecium	19434	1.5
20	Torulopsis glabrata	2001	0.9

Example 16

Members of the genus Proteus are motile, gram negative, non-sporeforming bacilli which belong in the family Enterobacteriaceae. Four species of Proteus have been described and three of them, Proteus mirabilis, P. vulgaris, and P. penneri, cause human disease.

The most common type of proteus infection involves the urinary tract, but septicemia, pneumonia and wound infections also occur. Proteus mirabilis is the species most often isolated and may account for up to 10% of all acute, uncomplicated urinary tract infections. Species, rather than genus level identification of the causative organism is desirable because of differential antibiotic susceptibility among the species.

The current method for identifying Proteus mirabilis

from patient samples involves culture of the specimen on agar plates for 18-24 hours, followed by a battery of biochemical tests. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately identifies Proteus mirabilis. The present invention reduces the number of tests which must be run on a sample, the time to identification and therefore, diagnosis and treatment. This represents a significant improvement over prior art methods.

The probe specific for Proteus mirabilis was obtained with a primer complementary to a conserved region of 23S rRNA with the sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'.

The following sequence was characterized and shown to be specific for P. mirabilis. The phylogenetically nearest neighbors Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris and Salmonella enteritidis were used as comparisons with the sequence of Proteus mirabilis.

1. CCG TTC TCC TGA CAC TGC TAT TGA TTA AGA CTC

This probe is capable of hybridizing to the RNA of P. mirabilis in the region corresponding to base 270-305 of E. coli 23S rRNA. The probe is 33 bases in length and has a T_m of 66°C. To demonstrate the reactivity and specificity of the probe for P. mirabilis, it was used in a hybridization assay. 32P-end-labeled oligonucleotide probe was mixed with RNA released from at least 10⁷ organisms in 1% sodium dodecyl sulfate, 0.48 M sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM EGTA (0.2 ml final volume) and incubated at 64°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 64°C for 10 minutes. The sample was centrifuged and the supernatant

removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant was removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 48 and demonstrate that the probe reacts well with P. mirabilis and does not react with 27 other closely related bacteria. Table 49 shows that the probe does not react with 24 other phylogenetically diverse bacteria and two yeasts tested in the same manner as the organisms in Table 48.

TABLE 48

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE
TO CLOSELY RELATED ORGANISMS

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
5	Citrobacter diversus	27156	1.1
	Citrobacter freundii	8090	1.1
	Citrobacter freundii	6750	1.0
10	Enterobacter aerogenes	13048	1.0
	Enterobacter agglomerans	27155	1.0
	Enterobacter cloacae	e13047	1.1
	Enterobacter gergoviae	33028	1.0
	Enterobacter sakazakii	29544	1.1
15	Escherichia coli	10798	1.2
	Escherichia coli	11775	1.2
	Escherichia coli	29417	1.2
	Klebsiella oxytoca	13182	1.0
	Klebsiella ozaenae	11296	1.1
20	Klebsiella planticola	33531	0.9
	Klebsiella pneumoniae	13883	1.3
	Klebsiella pneumoniae	23357	1.1
	Klebsiella rhinoscleromatis	13884	1.2
	Klebsiella terrigena	33257	1.1
25	Klebsiella trevisanii	33558	1.0
	Kluyvera ascorbata	33433	0.9
	Proteus mirabilis	25933	69.0
	Proteus penneri	33519	2.5
	Proteus vulgaris	13315	1.7
30	Providencia alcalifaciens	9886	1.1
	Providencia rettgeri	29944	1.3
	Providencia stuartii	29914	1.1
	Salmonella arizonae	29933	1.1
	Salmonella enteritidis	13076	0.8

TABLE 49

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE TO
PHYLOGENETICALLY DIVERSE ORGANISMS

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
40	Acinetobacter calcoaceticus	33604	0.8
	Bacillus subtilis	6051	1.2
	Bacteroides fragilis	23745	0.9
	Branhamella catarrhalis	25238	0.7
45	Campylobacter jejuni	33560	1.0
	Candida krusei	34135	0.8
	Chromobacterium violaceum	29094	1.1
	Clostridium perfringens	13124	0.9
	Deinococcus radiodurans	35073	0.8

	Derxia gummosa	15994	0.8
	Hafnia alvei	13337	0.9
	Morganella morganii	25830	0.9
	Pseudomonas aeruginosa	10145	1.0
5	Pseudomonas cepacia	17762	0.9
	Rahnella aquatilis	33071	0.9
	Rhodospirillum rubrum	11170	0.8
	Serratia marcescens	13880	0.9
	Serratia odorifera	33077	0.9
10	Staphylococcus aureus	e12600	0.8
	Staphylococcus epidermidis	14990	0.8
	Streptococcus mitis	9811	0.8
	Streptococcus pneumoniae	e6306	0.9
	Torulopsis glabrata	2001	0.9
15	Vibrio parahaemolyticus	17802	0.8
	Xanthomonas maltophilia	13637	1.1
	Yersinia enterocolitica	9610	0.8

Example 17

20 Members of the genus Salmonella are motile, gram negative, non-sporeforming bacilli which belong in the family Enterobacteriaceae. All salmonellae are highly related and some microbiologists consider them to be one species. Five subgroups have been identified using nucleic acid homology studies and over 25 1400 different serotypes have been described. All serotypes have been implicated in human enteric disease ranging from self-limited gastroenteritis with mild symptoms, to severe gastroenteritis with bacteremia, to typhoid fever, a potentially life-threatening illness. S. choleraesuis, S. paratyphi A and S. typhi are the serotypes most often associated with severe disease 30 and bacteremia. Diagnosis of Salmonella-induced enteritis is dependent upon detection of the organism in stool samples. Because infection occurs primarily by ingestion of contaminated milk, food and water, methods for identifying Salmonella in these 35 products before release to consumers is critical.

Current methods for detection of members of the genus Salmonella involve culture of the specimen for 1-3 days on selec-

tive media followed by a battery of biochemical tests. Often an enrichment step is needed to isolate *Salmonella* from clinical samples or food products. The oligonucleotide sequences shown below, when used in a hybridization assay, accurately identify members of the genus *Salmonella*. The present inventive probes are specific for all members of the genus and do not react with the other closely related *Enterobacteriaceae* genera. These inventive probes reduce the number of tests which must be run on a sample and greatly reduce the time to identification. This represents a significant improvement over prior art methods.

The probes specific for the genus *Salmonella* were obtained with two primers complementary to 16S and 23S rRNA. Sequence 1 was obtained using a 16S primer with the sequence 5' TTA CTA GCG ATT CCG ACT TCA 3'. Sequence 2 was obtained using a 23S primer with the sequence 5' CAG TCA GGA GTA TTT AGC CTT 3'. The following sequences were characterized and shown to be specific for the genus *Salmonella*:

1. CTC CTT TGA GTT CCC GAC CTA ATC GCT GGC
2. CTC ATC GAG CTC ACA GCA CAT GCG CTT TTG TGT A

Sequence 1, from 16S rRNA, is 30 bases in length and has a T_m of 73°C. Sequence 2, from 23S rRNA, is 34 bases long and has a T_m of 71°C. These probes are capable of hybridizing in the regions corresponding to bases 1125-1155 of *E. coli* 16S rRNA and 335-375 of *E. coli* 23S rRNA, respectively. To demonstrate the reactivity and specificity of probe 1 for members of the genus *Salmonella*, ³²P-end-labeled oligonucleotide was tested as a probe in a hybridization reaction. Purified RNA, or RNA released from at least 10⁷ organisms by standard methods, was mixed with 1 ml hybridization buffer (final concentration 43% diisobutyl

sulfosuccinate, ^{60 mM}~~50 mM~~ sodium phosphate pH 6.8, 1mM EDTA, 1mM EGTA) and incubated at 72°C for 2-12 hours. Following incubation, 5 ml of separation solution (2% hydroxyapatite, 0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added and the sample were mixed, incubated at 72°C for 5 minutes, centrifuged and the supernatants removed. Four ml of wash solution (0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate) was added and the samples were vortexed, centrifuged, and the supernatants removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results shown in Table 50 indicate that a combination of the two probes hybridized to the 5 subgroups of Salmonella and to all 31 of the serotypes which were tested.

TABLE 50

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2
 TO MEMBERS OF THE GENUS SALMONELLA

	Subgroup	Organism	ATCC#	% Probe Bound	
				Probe 1	Probe 2
20	I	Salmonella choleraesuis	10708	24	40
	I	Salmonella enteritidis	13076	15	67
	I	Salmonella paratyphi A	9150	1.4	70
	I	Salmonella sp. serotype anatum	9270	40	26
25	I	Salmonella sp. serotype cubana	12007	54	35
	I	Salmonella sp. serotype give	9268	12	40
	I	Salmonella sp. serotype heidelberg	8326	53	33
30	I	Salmonella sp. serotype illinois	11646	36	46
	I	Salmonella sp. serotype montevideo	8387	35	32
	I	Salmonella sp. serotype newington	29628	52	34
35	I	Salmonella sp. serotype newport	6962	3.4	36
	I	Salmonella sp. serotype putten	15787	34	39
40	I	Salmonella sp. serotype saintpaul	9712	28	30

	I	Salmonella sp. serotype senftenberg	8400	38	43
	I	Salmonella sp. serotype simsbury	12004	29	29
5	I	Salmonella sp. serotype sloterdijk	15791	34	30
	I	Salmonella sp. serotype thompson	8391	32	41
10	I	Salmonella sp. serotype vellore	15611	35	2.6
	I	Salmonella typhi	19430	7.0	21
	I	Salmonella typhimurium	14028	69	69
	II	Salmonella salamae	6959	3.0	46
15	II	Salmonella sp. serotype maarsen	15793	6.6	30
	III	Salmonella arizonae	33952	2.9	38
	III	Salmonella arizonae	12324	5.5	42
	III	Salmonella arizonae	29933	2.3	62
	III	Salmonella arizonae	29934	63	12
20	III	Salmonella arizonae	12323	4.0	39
	III	Salmonella arizonae	12325	51	1.9
	IV	Salmonella sp. serotype harmelen	15783	5.8	8.0
25	IV	Salmonella sp. serotype ochsenzoll	29932	7.5	40
	V	Salmonella sp. serotype bongor	cdc1319	60	1.8

The specificity of the probes for members of the genus Salmonella was demonstrated with hybridization reactions containing RNA from organisms closely related to Salmonella. The results are shown in Table 51.

TABLE 51

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2
TO RNA OF CLOSELY RELATED ORGANISMS

	Organism	ATCC#	% Probe Bound	
			Probe 1	Probe 2
	Citrobacter freundii	6750	2.2	0
40	Edwardsiella tarda	15947	0	0
	Enterobacter agglomerans	27155	0.6	0
	Enterobacter cloacae	13047	0	0
	Enterobacter sakazakii	29544	0	0
	Escherichia coli	10798	0	0
45	Escherichia coli	29417	0	0
	Klebsiella pneumoniae	23357	0.7	0

Kluyvera ascorbata	33433	0	0.5
Proteus mirabilis	25933	0.2	0
Shigella flexneri	29903	0	0

5 *% Probe Bound = counts bound to hydroxyapatite - counts bound
when no RNA present/total counts used in assay

Table 52 shows that Salmonella probes 1 and 2 do not hybridize to phylogenetically diverse organisms.

10

TABLE 52

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2 TO
RNA OF A PHYLOGENETIC CROSS SECTION OF ORGANISMS

Organism		ATCC#	% Probe Bound* Probe 1 and Probe 2	
15	Acinetobacter calcoaceticus	33604	1.1	0.1
	Bacillus subtilis	6051	0	0.5
	Bacteroides fragilis	23745	0.1	0
	Branhamella catarrhalis	25238	0.9	0
	Campylobacter jejuni	33560	0	0.2
20	Candida krusei	34135	0.4	0.3
	Chromobacterium violaceum	29094	1.7	0
	Clostridium perfringens	13124	0.3	0
	Deinococcus radiodurans	35073	1.6	0.1
	Derxia gummosa	15994	1.2	0
25	Hafnia alvei	13337	1.8	0
	Morganelli morganii	25830	0	1.1
	Pseudomonas aeruginosa	10145	0.5	0.7
	Pseudomonas cepacia	17762	0	0
	Pseudomonas maltophilia	13637	1.9	0
30	Rahnella aquatilis	33071	1.2	0.3
	Rhodospirillum rubrum	11170	0.9	0
	Serratia marcescens	13880	0	0
	Serratia odorifera	33077	2.6	0.2
	Staphylococcus aureus	e12600	0.2	0
35	Staphylococcus epidermidis	14990	0	0
	Streptococcus nitris	9811	1.2	0.7
	Streptococcus pneumoniae	e6306	0	0
	Torulopsis glabrata	2001	0	0
	Vibrio parahaemolyticus	17802	0	0.2
40	Yersinia enterocolitica	9610	0	0

*% Probe Bound = Counts bound to hydroxyapatite - counts bound
when no RNA present/total counts used in assay

Example 18

Escherichia coli is a gram negative, nonsporeforming bacillus which belongs in the family Enterobacteriaceae. Five species of Escherichia have been described: E. coli, which
5 accounts for >99% of the clinical isolates, E. hermannii, E. blattae, E. vulneris and E. fergusonii. E. coli is a leading cause of urinary tract infections, bacteremia and neonatal meningitidis, and can cause a type of gastroenteritis known as traveller's diarrhea.

10 The current method for identifying E. coli from patient samples involves culture of the specimen on agar plates for 18-72 hours, followed by a battery of biochemical tests on isolated colonies. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately
15 detects E. coli even in the presence of other organisms. The present invention reduces the number of tests which must be run on a sample and reduces the time to identification and therefore diagnosis and treatment. This represents a significant improvement over prior art methods.

20 The probe specific for E. coli was derived from the published E. coli sequence (Brosius, et al. Proc. Natl. Acad. Sci. U.S.A. 75:4801-4805 (1978)), using Proteus vulgaris (Carbon, et al., Nuc. Acids Res. 9:2325-2333 (1981)), Klebsiella pneumoniae, Salmonella enteritidis, Enterobacter gergoviae and Citrobacter freundii for comparison. The probe sequence is shown below.
25

1. GCA CAT TCT CAT CTC TGA AAA CTT CCG TGG

It hybridizes to RNA of E. coli in the region of 995-
1030 of ^{16S} ~~26S~~ rRNA. The probe is 30 bases in length and has a T_m
of 66°C. To demonstrate the reactivity and specificity of the

probe for *E. coli*, it was used in a hybridization assay. ³²P-
end-labeled oligonucleotide probe was mixed with two unlabeled
oligonucleotides of ^{Sequences} ~~sequence~~ 5'-TGG ATG TCA AGA CCA GGT AAG GTT
CTT CGC GTT GCA TCG-3' and 5'-CTG ACG ACA GCC ATG CAG CAC CTG TCT
CAC GGT TCC CGA AGG CA-3' and with purified RNA, or RNA released
from cells with detergent and heat, in 1% sodium dodecyl sulfate
(SDS), 0.48 M sodium phosphate pH 6.8, ^{1 mM} ~~1 mM~~ EDTA, 1 mM EGTA (0.2
ml final volume) and incubated at 60°C for 2 hours. Following
incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH
6.8, 0.02% sodium dodecyl sulfate was added and the mixture
incubated at 60°C for 10 minutes. The sample was centrifuged and
the supernatant removed. Five ml of wash solution (0.12 M sodium
phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the
sample vortexed, centrifuged and the supernatant was removed.
The amount of radioactivity bound to the hydroxyapatite was
determined by scintillation counting.

An example of a use for this probe would be to detect
E. coli in urine samples. Table 53 shows that the probe detects
7 out of 8 strains of *E. coli* tested. The probe also reacts with
E. fergusonii, an organism which would only rarely be found in
urine.

Table 54 shows that the probe does not react with any
other genus tested except *Shigella*, another organism rarely
isolated from urine. These results show that the probe will be
useful in detecting *E. coli* from urine samples.

TABLE 53

HYBRIDIZATION OF E. coli TO ESCHERICHIA SPECIES

5	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Escherichia coli	10798	70
	E. coli	11775	67
	E. coli	23722	58
	E. coli	25404	68
10	E. coli	25922	55
	E. coli	29417	72
	E. coli	33780	0.8
	E. coli	35150	45
	E. fergusonii	35469	55
15	E. hermannii	33650	0.7
	E. vulneris	33821	0.8

TABLE 54

HYBRIDIZATION OF THE E. coli PROBE TO
CLOSELY RELATED ORGANISMS

20	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Citrobacter freundii	6750	0.8
	Citrobacter freundii	8090	0.9
	Citrobacter freundii	29221	0.6
25	Citrobacter freundii	33128	0.6
	Enterobacter aerogenes	13048	1.2
	Enterobacter agglomerans	27155	0.9
	Enterobacter cloacae	13047	0.9
	Enterobacter gergoviae	33023	0.7
30	Enterobacter sakazakii	29544	0.6
	Klebsiella oxytoca	13182	0.7
	Klebsiella pneumoniae	13883	0.7
	Proteus mirabilis	29906	0.7
	Proteus vulgaris	13315	0.8
35	Shibella boydii	8700	76
	Shigella dysenteriae	13313	0.8
	Shigella flexneri	29903	71
	Shigella sonnei	29930	75

Example 19

The bacteria encompass a morphologically and physiologically diverse group of unicellular organisms which occupy most natural environments. Although many bacteria are harmless or beneficial to their environment or host, some are

harmful and cause disease. The presence of any bacteria in some locations is undesirable or indicative of disease (e.g., culture media, pharmaceutical products, body fluids such as blood, urine or cerebrospinal fluid, and tissue biopsies). Low levels of
5 bacteria are considered acceptable in other products such as drinking water and food products. Accordingly, there is a need for a means for detecting and quantitating bacteria in a sample.

The current method of detection and quantitation of total bacteria in a sample requires culture on multiple types of
10 media under different conditions of temperature and atmosphere. To date, no single test exists to detect or quantitate all bacteria. The oligonucleotide sequences shown below, when used in a hybridization assay, detect a broad phylogenetic cross section of bacteria. The present invention reduces the number of
15 tests which need to be performed and also reduces the time required for the assay. Comparison of the hybridization results from an unknown sample to a set of standards will allow some quantitation of the number of bacteria present. This represents a significant improvement over prior art methods.

20 The bacterial probes were designed following examination of published sequences of rRNA and sequences determined at Gen-Probe. The sequences used for the comparison include Agrobacterium tumefaciens (Yang et al., Proc. Natl. Acad. Sci. U.S.A., 82:4443, (1985), Anacystis nidulans (Tomiooka and
25 Sugiura. Mol. Gen. Genet. 191:46, (1983), Douglas and Doolittle Nuc. Acids Res. 12:3373, (1984), Bacillus subtilis (Green et al., Gene 37:261. (1985), Bacillus stearothermophilus (Kop et al., DNA 3:347, (1984), Bacteroides fragilis (Weisburg et al., J. Bacteriol. 164:230, (1985), Chlamydia psittaci (Weisburg et al.,

J. Bacteriol. 167:570. (1986)), Desulfovibrio desulfuricans (Oyaizu and Woese, System. Appl. Microbiol. 6:257, (1985); Escherichia coli, (Brosius et al., Proc. Natl. Acad. Sci. U.S.A. 77:201, (1980); Flavobacterium heparinum (Weisburg et al., J. Bacteriol. 164:230, (1985); Hellobacterium chlorum (Woese et al., Science 229:762, (1985); Mycoplasma PG50 (Frydenberg and Christiansen, DNA 4:127, (1985); Proteus vulgaris (Carbon et al., Nuc. Acids Res. 9:2325, (1981); Pseudomonas testosteroni (Yang et al., Proc. Natl. Acad. Sci. U.S.A. 82:4443, (1985); Rochalimaea quintana (Weisburg et al., Science 230:556, (1985); Saccharomyces cerevisiae (Rubstov et al., Nuc. Acids Res. 8:5779, (1980); Georgiev et al., Nuc. Acids Res. 9:6953, (1981); and human (Torczynski et al., DNA 4:283, (1985); Gonzalez et al., Proc. Natl. Acad. Sci. U.S.A. 82:7666, (1985)).

The following sequences were shown to hybridize to a broad phylogenetic cross section of bacteria and not to yeast or human rRNA:

1. CCA CTG CTG CCT CCC GTA GGA GTC TGG GCC
2. CCA GAT CTC TAC GCA TTT CAC CGC TAC ACG TGG
3. GCT CGT TGC GGG ACT TAA CCC AAC AT
4. GGG GTT CTT TTC GCC TTT CCC TCA CGG
5. GGC TGC TTC TAA GCC AAC ATC CTG
6. GGA CCG TTA TAG TTA CGG CCG CC
7. GGT CGG AAC TTA CCC GAC AAG GAA TTT CGC TAC C

Probe 1 is 30 bases long and has a Tm of 70°C. Probe 2 is 33 bases long and has a Tm of 69°C. Probe 3 is 26 bases long and has a Tm of 67°C. Probe 4 is 27 bases long and has a Tm of 69°C. Probe 5 is 24 bases long and has a Tm of 66°C. Probe 6 is 23 bases long and has a Tm of 62°C. Probe 7 is 34 bases long and

has a T_m of 66°C. Probes 1-3 hybridize to 16S rRNA in the following regions, respectively, (corresponding to E. coli bases) 330-365; 675-715; and 1080-1110. Probes 4-7 hybridize to 23S rRNA in the following regions, respectively, (corresponding to E. coli bases) 460-490; 1050-1080; and 1900-1960 (probes 6 and 7). The oligonucleotides interact with regions on the rRNA which are highly conserved among eubacteria. This means that they can be used as bacterial probes in a hybridization assay. A second use is as a tool to obtain rRNA sequence. For example, an oligonucleotide can be hybridized to the rRNA of interest and extended with reverse transcriptase. The sequence of the resulting DNA can be determined and used to deduce the complementary rRNA sequence as described in the Detailed Description of the Invention.

One application of the invention is to detect bacteria in urine (bacteriuria). To demonstrate the reactivity and specificity of the probes for bacteria found in urine, they were used in hybridization assays. ^{32}P -end-labeled or ^{125}I -labeled oligonucleotide probes were mixed with RNA released from cells by standard methods (e.g., the sonic disruption techniques described in Murphy et al., U.S. Patent ^{5,374,522} ~~App. Ser. No. 841,060~~, detergent with glass beads, or enzymatic lysis). Probe was mixed with RNA in 0.48 M sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM EGTA, 1% sodium dodecyl sulfate (0.2 ml final volume) and hybridized at 60°C for 2 hours. Five ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 60°C for 10 minutes. The mixture was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl

sulfate) was added and the sample was mixed, centrifuged and the supernatant removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. Tables 55-68 demonstrate the specificity of these probes and show that a combination of probes could be used to detect all bacteria which have been tested.

Table 55 shows that probe 1 hybridizes to the RNA of bacteria commonly isolated from urine and does not detect yeast RNA. Table 56 shows that probe 1 detects phylogenetically diverse bacteria and does not hybridize to human RNA.

TABLE 55

HYBRIDIZATION OF BACTERIAL PROBE 1
TO RNA OF ORGANISMS FOUND IN URINE

	Organism	ATCC#	% Probe* Bound
15	Candida albicans	18804	2.6
	Candida krusei	34135	2.2
	Candida parapsilosis	22019	2.9
20	Candida tropicalis	750	2.5
	Citrobacter freundii	8090	69
	Enterobacter aerogenes	13048	70
	Enterobacter cloacae	13047	71
	Escherichia coli	11775	67
25	Klebsiella oxytoca	13182	70
	Klebsiella pneumoniae	13883	72
	Morganella morganii	25830	66
	Proteus mirabilis	29906	71
	Proteus vulgaris	13315	67
30	Providencia stuartii	29914	69
	Pseudomonas aeruginosa	10145	76
	Pseudomonas fluorescens	13525	73
	Serratia marcescens	13880	66
	Staphylococcus aureus	12600	57
35	Staphylococcus epidermidis	14990	68
	Streptococcus agalactiae	13813	68
	Streptococcus faecalis	19433	51
	Streptococcus faecium	19434	53
	Torulopsis glabrata	2001	2.3
40	Ureaplasma urealyticum	27618	54

TABLE 56

HYBRIDIZATION OF BACTERIAL PROBE 1 TO RNAs

~~OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS.~~

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe*</u>
			<u>Bound</u>
	Acinetobacter calcoaceticus	23055	65
	Bacillus subtilis	6051	73
10	Bacteroides fragilis	23745	61
	Branhamella catarrhalis	25238	72
	Campylobacter jejuni	33560	64
	Chlamydia trachomatis	VR878	14
	Chromobacterium violaceum	29094	71
15	Clostridium perfringens	13124	74
	Corynebacterium xerosis	373	38
	Deinococcus radiodurans	35073	47
	Derxia gummosa	15994	65
	Gardnerella vaginalis	14018	67
20	Hafnia alvei	13337	60
	Lactobacillus acidophilus	4356	56
	Moraxella osloensis	19976	61
	Mycobacterium smegmatis	14468	47
	Mycoplasma hominis	14027	58
25	Neisseria gonorrhoeae	19424	58

Rahnella aquatilis	33071	74
Rhodospirillum rubrum	11170	73
Vibrio parahaemolyticus	17802	75
Human		2.5

5

Table 57 shows that Probe 2 hybridizes to the RNA of bacteria commonly found in urine except Ureaplasma urealyticum and does not hybridize to yeast rRNA.

TABLE 57

10

HYBRIDIZATION OF BACTERIAL PROBE 2
TO RNA OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>%Probe* Bound</u>
	Candida albicans	18804	2.5
15	Candida krusei	34135	1.8
	Candida parapsilosis	22019	1.6
	Candida tropicalis	750	1.4
	Citrobacter freundii	8090	61
	Enterobacter aerogenes	13048	57
20	Enterobacter cloacae	13047	61
	Escherichia coli	11775	67
	Klebsiella oxytoca	13182	67
	Klebsiella pneumoniae	13883	51
	Morganella morganii	25830	69
25	Proteus mirabilis	29906	69
	Proteus vulgaris	13315	69
	Providencia stuartii	29914	66
	Pseudomonas aeruginosa	10145	59
	Pseudomonas fluorescens	13525	58
30	Serratia marcescens	13880	64
	Staphylococcus aureus	12600	60
	Staphylococcus epidermidis	14990	60
	Streptococcus agalactiae	13813	54
	Streptococcus faecalis	19433	37
35	Streptococcus faecium	19434	58
	Torulopsis glabrata	2001	1.5
	Ureaplasma urealyticum	27618	3.2

40 Table 58 shows that probe 2 detects phylogenetically diverse

bacteria and does not hybridize to human rRNA.

TABLE 58

HYBRIDIZATION OF BACTERIAL PROBE 2 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS/

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe*</u> <u>Bound</u>
	Acinetobacter calcoaceticus	23055	76
	Bacillus subtilis	6051	75
10	Bacteroides fragilis	23745	2.0
	Branhamella catarrhalis	25238	70
	Campylobacter jejuni	33560	2.5
	Chlamydia trachomatis	VR878	16
	Chromobacterium violaceum	29094	61
15	Clostridium perfringens	13124	66
	Corynebacterium xerosis	373	3.8
	Deinococcus radiodurans	35073	6.0
	Derxia gummosa	15994	61
	Gardnerella vaginalis	14018	2.0
20	Hafnia alvei	13337	72
	Lactobacillus acidophilus	4356	50
	Moraxella osloensis	19976	64
	Mycobacterium smegmatis	14468	19
	Mycoplasma hominis	14027	34
25	Neisseria gonorrhoeae	19424	71
	Rahnella aquatilis	33071	77
	Rhodospirillum rubrum	11170	1.5
	Vibrio parahaemolyticus	17802	73
	Yersinia enterocolitica	9610	76
30	Human		2.0

Table 59 shows that probe 3 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 59

HYBRIDIZATION OF BACTERIAL PROBE 3 TO RNA OF
ORGANISMS FOUND IN URINE/

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe*</u> <u>Bound</u>
40	Candida albicans	18804	1.4
	Candida krusei	34135	1.5
	Candida parapsilosis	22019	2.2
	Candida tropicalis	750	2.6
	Citrobacter freundii	8090	79

	Enterobacter aerogenes	13048	40
	Enterobacter cloacae	13047	44
	Escherichia coli	11775	67
	Klebsiella oxytoca	13182	38
5	Klebsiella pneumoniae	13883	45
	Morganella morganii	25830	57
	Proteus mirabilis	29906	40
	Proteus vulgaris	13315	51
	Providencia stuartii	29914	54
10	Pseudomonas aeruginosa	10145	61
	Pseudomonas fluorescens	13525	56
	Serratia marcescens	13880	54
	Staphylococcus aureus	12600	37
	Staphylococcus epidermidis	14990	20
15	Streptococcus agalactiae	13813	34
	Streptococcus faecalis	19433	20
	Streptococcus faecium	19434	47
	Torulopsis glabrata	2001	1.9
	Ureaplasma urealyticum	27618	26

Table 60 shows that probe 3 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

TABLE 60

25 HYBRIDIZATION OF BACTERIAL PROBE 3 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS/

	Organism Name	ATCC#	% Probe Bound
	Acinetobacter calcoaceticus	23055	69
30	Bacillus subtilis	6051	35
	Bacteroides fragilis	23745	1.2
	Branhamella catarrhalis	25238	43
	Campylobacter jejuni	33560	55
	Chlamydia trachomatis	VR878	42
35	Chromobacterium violaceum	29094	69
	Clostridium perfringens	13124	62
	Corynebacterium xerosis	373	23
	Deinococcus radiodurans	35073	30
	Derxia gummosa	15994	67
40	Gardnerella vaginalis	14018	40
	Hafnia alvei	13337	56
	Lactobacillus acidophilus	4356	36
	Moraxella osloensis	19976	64
	Mycobacterium smegmatis	14468	77
45	Mycoplasma hominis	14027	1.5
	Neisseria gonorrhoeae	19424	26
	Rahnella aquatilis	33071	66
	Rhodospirillum rubrum	11170	51
	Vibrio parahaemolyticus	17802	68
50	Yersinia enterocolitica	9610	68
	Human		0.9

Table 61 shows that probe 4 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 61

HYBRIDIZATION OF BACTERIAL PROBE 4 TO RNA OF
ORGANISMS FOUND IN URINE/

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Candida albicans	18804	4.5
	Candida krusei	34135	2.5
	Candida parapsilosis	22019	2.7
	Candida tropicalis	750	2.5
	Citrobacter freundii	8090	55
15	Enterobacter aerogenes	13048	52
	Enterobacter cloacae	13047	57
	Escherichia coli	11775	70
	Klebsiella oxytoca	13182	70
	Klebsiella pneumoniae	13883	43
20	Morganella morganii	25830	74
	Proteus mirabilis	29906	74
	Proteus vulgaris	13315	73
	Providencia stuartii	29914	73
	Pseudomonas aeruginosa	10145	76
25	Pseudomonas fluorescens	13525	79
	Serratia marcescens	13880	74
	Staphylococcus aureus	12600	73
	Staphylococcus epidermidis	14990	73
	Streptococcus agalactiae	13813	70
30	Streptococcus faecalis	19433	37
	Streptococcus faecium	19434	63
	Torulopsis glabrata	2001	2.2
	Ureaplasma urealyticum	27618	43

Table 62 shows that probe 4 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

TABLE 62

HYBRIDIZATION OF BACTERIAL PROBE 4 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE
ORGANISMS

% Probe

	<u>Organism Name</u>	<u>ATCC#</u>	<u>Bound</u>
	Acinetobacter calcoaceticus	23055	69
	Bacillus subtilis	6051	55
	Bacteroides fragilis	23745	3.0
5	Branhamella catarrhalis	25238	59
	Campylobacter jejuni	33560	65
	Chlamydia trachomatis	VR878	50
	Chromobacterium violaceum	29094	61
	Clostridium perfringens	13124	57
10	Corynebacterium xerosis	373	9.5
	Deinococcus radiodurans	35073	63
	Derxia gummosa	15994	65
	Gardnerella vaginalis	14018	57
	Hafnia alvei	13337	67
15	Lactobacillus acidophilus	4356	68
	Moraxella osloensis	19976	68
	Mycobacterium smegmatis	14468	28
	Mycoplasma hominis	14027	74
	Neisseria gonorrhoeae	19424	76
20	Rahnella aquatilis	33071	68
	Rhodospirillum rubrum	11170	59
	Vibrio parahaemolyticus	17802	75
	Yersinia enterocolitica	9610	74
25	Human		2.8

Table 63 shows that probe 5 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 63
HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNA OF
ORGANISMS FOUND IN URINE/

Organism	ATCC#	% Probe Bound
Candida albicans	18804	1.8
Candida krusei	34135	1.7
Candida parapsilosis	22019	2.2
Candida tropicalis	750	1.8
Citrobacter freundii	8090	39
Enterobacter aerogenes	13048	38
Enterobacter cloacae	13047	43
Escherichia coli	11775	31
Klebsiella oxytoca	13182	38
Klebsiella pneumoniae	13883	66
Morganella morganii	25830	50
Proteus mirabilis	29906	44
Proteus vulgaris	13315	52
Providencia stuartii	29914	44
Pseudomonas aeruginosa	10145	47
Pseudomonas fluorescens	13525	25
Serratia marcescens	13880	35
Staphylococcus aureus	12600	26
Staphylococcus epidermidis	14990	37
Streptococcus agalactiae	13813	29
Streptococcus faecalis	19433	14
Streptococcus faecium	19434	33
Torulopsis glabrata	2001	2.2
Ureaplasma urealyticum	27618	73

Table 64 shows that probe 5 detects phylogenetically diverse bacteria and does not hybridize to human RNA.

TABLE 64
HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE
ORGANISMS

Organism	ATCC#	% Probe Bound
Acinetobacter calcoaceticus	23055	20
Bacillus subtilis	6051	53
Bacteroides fragilis	23745	44
Branhamella catarrhalis	25238	22
Campylobacter jejuni	33560	35
Chromobacterium violaceum	29094	59
Clostridium perfringens	13124	63

	Corynebacterium xerosis	373	1.7
	Deinococcus radiodurans	35073	5.7
	Derxia gummosa	15994	14
	Gardnerella vaginalis	14018	1.6
5	Hafnia alvei	13337	44
	Lactobacillus acidophilus	4356	1.5
	Moraxella osloensis	19976	7.2
	Mycobacterium smegmatis	14468	39
	Mycoplasma hominis	14027	21
10	Neisseria gonorrhoeae	19424	40
	Rahnella aquatilis	33071	55
	Rhodospirillum rubrum	11170	17
	Vibrio parahaemolyticus	17802	66
	Yersinia enterocolitica	9610	64
15	Human		1.6

Table 65 shows that probe 6 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

20

TABLE 65

HYBRIDIZATION OF BACTERIAL PROBE 6 TO RNA OF
ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
25	Candida albicans	18804	3.0
	Candida krusei	34135	2.0
	Candida parapsilosis	22019	2.2
	Citrobacter freundii	8090	54
	Enterobacter aerogenes	13048	50
30	Enterobacter cloacae	13047	58
	Escherichia coli	11775	63
	Klebsiella oxytoca	13182	54
	Klebsiella pneumoniae	13883	55
	Morganella morganii	25830	60
35	Proteus mirabilis	29906	64
	Proteus vulgaris	13315	67
	Providencia stuartii	29914	64
	Pseudomonas aeruginosa	10145	65
	Pseudomonas fluorescens	13525	31
40	Serratia marcescens	13880	67
	Staphylococcus aureus	12600	53
	Staphylococcus epidermidis	14990	34
	Streptococcus agalactiae	13813	31
	Streptococcus faecium	19434	18
45	Torulopsis glabrata	2001	2.5

Table 66 shows that probe 6 detects some phylogenetically diverse bacteria and does not hybridize to human rRNA.

TABLE 66

HYBRIDIZATION OF BACTERIAL PROBE 6 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY
DIVERSE ORGANISMS/

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Acinetobacter calcoaceticus	23055	73
	Bacteroides fragilis	23745	7.0
	Branhamella catarrhalis	25238	4.0
	Deinococcus radiodurans	35073	5.5
	Derxia gummosa	15994	3.0
15	Gardnerella vaginalis	14018	2.0
	Hafnia alvei	13337	3.5
	Lactobacillus acidophilus	4356	17
	Moraxella osloensis	19976	62
	Mycoplasma hominis	14027	44
20	Rahnella aquatilis	33071	56
	Yersinia enterocolitica	9610	50
	Human		4.0

Table 67 shows that probe 7 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 67

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNA
OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
30	Candida albicans	18804	2.1
	Candida krusei	34135	2.0
	Candida tropicalis	750	2.2
35	Citrobacter freundii	8090	67
	Enterobacter aerogenes	13048	69
	Enterobacter cloacae	13047	78
	Escherichia coli	11775	75
	Klebsiella oxytoca	13882	79
40	Klebsiella pneumoniae	13883	77
	Morganella morganii	25830	76
	Proteus mirabilis	29906	77

	Proteus vulgaris	13315	79
	Providencia stuartii	29914	64
	Pseudomonas aeruginosa	10145	76
	Pseudomonas fluorescens	13525	78
5	Serratia marcescens	13880	66
	Staphylococcus aureus	12600	71
	Staphylococcus epidermidis	14990	75
	Streptococcus agalactiae	13813	70
	Streptococcus faecalis	19433	58
10	Streptococcus faecium	19434	68
	Torulopsis glabrata	2001	2.4
	Ureaplasma urealyticum	27618	21

Table 68 shows that probe 7 detects phylogenetically diverse
bacteria and does not hybridize to human rRNA.

TABLE 68

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY
DIVERSE ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	23055	86
	Bacillus subtilis	6051	83
25	Bacteroides fragilis	23745	69
	Branhamella catarrhalis	25238	74
	Campylobacter jejuni	33560	5.3
	Chlamydia trachomatis	VR878	41
	Chromobacterium violaceum	29094	69
30	Clostridium perfringens	13124	68
	Corynebacterium xerosis	373	23
	Deinococcus radiodurans	35073	70
	Derxia gummosa	15994	69
	Gardnerella vaginalis	14018	68
35	Hafnia alvei	13337	77
	Moraxella osloensis	19976	68
	Mycobacterium smegmatis	14468	64
	Mycoplasma hominis	14027	4.0
	Neisseria gonorrhoeae	19424	53
40	Rahnella aquatilis	33071	72
	Rhodospirillum rubrum	11170	73
	Vibrio parahaemolyticus	17802	67
	Yersinia enterocolitica	9610	66
45	Human		2.2

Example 20

Fungi encompass a morphologically and physiologically diverse group of simple eucaryotic organisms. We estimate, using published sequences of three fungi, Neurospora crassa, Podospora, and Saccharomyces, that the rRNA of fungi are 58-60% homologous to E. coli and 84-90% homologous to one another. Some fungi grow as single cells (yeasts), others as multinuclear filaments (molds) and still others can grow as either single cells or multicellular filaments (dimorphic fungi). Although many fungi are harmless inhabitants of their environments, others are harmful and cause disease. The presence of any fungi in some locations is undesirable or indicative of disease (e.g., culture media, pharmaceutical products, body fluids such as blood, urine or cerebrospinal fluid, and tissue biopsies). Low levels of fungi are considered acceptable in other products such as drinking water and food products. This has created the need for a means of detecting and quantitating fungi in a sample.

The current methods for detecting and quantifying fungi involve microscopic examination of samples and culture on different media. Although most yeasts can be grown from clinical samples in a matter of days, some filamentous fungi take up to four weeks culture time, after which special staining procedures, biochemical analysis and antigen tests are performed. The oligonucleotide sequences below, when used in a hybridization assay, detect the five yeasts most commonly isolated in the clinical setting, Candida albicans, Torulopsis glabrata, Candida tropicalis, Candida parapsilosis and Candida krusei. Five other fungi representing the Trichosporon, Blastomyces, Cryptococcus and Saccharomyces genera are also detected. The present

invention allows one step detection of these organisms and, in relation to culture, reduces the time to identification or elimination of these fungi as the cause of an infection. This represents a significant improvement over prior art methods.

5 The four probes which hybridize to the organisms of interest were identified using 3 primers complementary to conserved regions on 18S or 28S rRNA. Sequence 1 was obtained using an 18S primer with the sequence 5'-AGA ATT TCA CCT CTG-3'. Sequence 2 was obtained using a 28S primer with the sequence 5'-
10 CCT TCT CCC GAA GTT ACG G-3'. Sequences 3 and 4 were obtained with a 28S primer with the sequence 5'-TTC CGA CTT CCA TGG CCA CCG TCC-3'. The following sequences were characterized and shown to hybridize to fungal rRNA. The sequences of Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Escherichia coli and
15 human rRNA were used for comparison with the sequences of interest.

1. CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG
2. CGA CTT GGC ATG AAA ACT ATT CCT TCC TGT GG
3. GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC AAC
20 AAG G
4. GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA AAC
AGG G

Sequence 1, from 18S rRNA, is 30 bases in length and has a T_m of 68°C. Sequence 2, from 23S rRNA, is 32 bases in
25 length and has a T_m of 67°C. Sequence 3, from 23S rRNA, is 40 bases in length and has a T_m of 66°C. Sequence 4, from 23S rRNA, is 40 bases in length and has a T_m of 68°C. Sequence 1 hybridizes in the region corresponding to position 845-880 of Saccharomyces cerevisiae ^{18S} ~~18S~~ rRNA. Sequence 2 hybridizes in the

region corresponding to position 1960-2000 of Saccharomyces
cerevisiae ^{28S} ~~28S~~ rRNA and sequences 3 and 4 hybridize in the region
of 1225-1270 of the ^{28S} ~~28S~~ rRNA.

To demonstrate the reactivity and specificity of these
probes for fungal RNA, they were used in hybridization assays.
32p- or 125I-labeled oligonucleotide probes were mixed with
purified RNA or RNA released from cells by standard lysis
techniques in 0.2 ml of ^{0.48 M} ~~0.48 M~~ sodium phosphate pH 6.8, 1% sodium
dodecyl sulfate, ^{1 mM} ~~1 mM~~ EDTA, ^{1 mM} ~~1 mM~~ EGTA and incubated at 60°C for 2
hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M
sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added
and the samples incubated 10 minutes at 60°C. The samples were
centrifuged and the supernatants removed. Five ml of ^{0.12 M} ~~0.12 M~~
sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added,
the samples were mixed, centrifuged and the supernatants removed.
The results are shown in Table 69. Probe 1 detects all ten fungi
which were tested, probe 2 detects all six of the yeasts which
were tested, probe 3 detects five of the six yeasts, and probe 4
detects C. krusei only. Thus probe 4 could be used to detect and
identify C. krusei in samples, ^{probes} ~~probe~~ 1, 2, or ^{a combination} ~~combination~~ of 3 and
4 could be used to detect the yeasts, and probe 1 could be used
to detect any of the ten organisms listed in Table 69.

One potential use for these probes is to identify
yeasts in urine samples or other normally sterile body fluids.
The probes were hybridized to a panel of bacteria most commonly
isolated from urine and shown not to react (Table 70). Table 71
shows that the probes do not hybridize to phylogenetically
diverse bacteria or to human RNA.

TABLE 69

HYBRIDIZATION OF YEAST PROBES TO YEAST RNA

5	Organism	ATCC#	% Probe Bound			
			#1	#2	#3	#4
	Blastomyces dermatitidis	C.I.	25	1.4	1.5	1.5
	Candida albicans	18804	40	63	56	2.0
	C. krusei	34135	73	62	2.2	70
	C. parapsilosis	22019	71	63	65	2.0
10	C. tropicalis	750	62	71	71	2.0
	Cryptococcus laurentii	C.I.	43	1.4	1.5	1.5
	Cryptococcus neoformans	C.I.	60	1.3	1.5	1.6
	Torulopsis glabrata	2001	61	44	62	2.0
	Trichosporon beigellii	C.I.	57	1.3	2.1	1.5
15	Saccharomyces cerevisiae	C.I.	41	67	53	1.9

C.I. = Clinical isolate

TABLE 70

HYBRIDIZATION OF FUNGAL PROBES 1-4 TO RNA
OF ORGANISMS FOUND IN URINE

20	Organism	ATCC#	% Probe Bound			
			#1	#2	#3	#4
	Citrobacter freundii	8090	1.5	1.7	1.5	2.1
	Enterobacter aerogenes	13048	2.5	1.9	2.0	2.0
25	Enterobacter cloacae	13047	2.5	1.6	2.6	2.0
	Escherichia coli	11775	3.0	2.0	1.6	1.5
	Klebsiella oxytoca	13182	2.5	2.2	2.5	2.0
	Klebsiella pneumoniae	13883	2.5	2.2	2.1	2.0
	Morganella morganii	25830	2.0	2.8	1.7	1.9
30	Proteus mirabilis	29906	2.5	1.9	2.3	2.0
	Proteus vulgaris	13315	2.0	2.2	2.0	1.5
	Providencia stuartii	29914	3.0	1.7	2.8	2.0
	Pseudomonas aeruginosa	10145	2.0	1.9	1.3	2.0
	Pseudomonas fluorescens	13525	2.5	2.7	2.1	2.0
35	Serratia marcescens	13880	2.5	1.7	1.8	2.0
	Staphylococcus aureus	12600	2.0	1.7	1.8	2.0
	Staphylococcus epidermidis	14990	3.0	1.5	1.3	2.0
	Streptococcus agalactiae	13813	2.5	1.9	1.3	2.5
	Streptococcus faecalis	19433	1.7	3.3	3.5	1.9
40	Streptococcus faecium	19434	2.0	2.9	2.1	1.5
	Ureaplasma urealyticum	27618	2.1	3.1	2.4	1.8

TABLE 71

HYBRIDIZATION OF FUNGAL PROBES 1-4 TO RNAs OF A CROSS
SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS

	Organism	ATCC#	% Probe Bound			
			#1	#2	#3	#4
5	Acinetobacter calcoaceticus	23055	2.5	2.5	2.0	1.9
	Bacillus subtilis	6051	2.0	2.8	2.4	2.4
	Bacteroides fragilis	23745	2.0	2.2	2.5	2.3
10	Branhamella catarrhalis	25238	2.5	3.2	1.8	1.7
	Campylobacter jejuni	33560	2.5	2.1	2.0	1.9
	Chlamydia trachomatis	VR878	3.1	3.1	1.8	2.7
	Chromobacterium violaceum	29094	2.5	1.7	2.0	2.2
	Clostridium perfringens	13124	1.9	2.3	1.8	1.8
15	Corynebacterium xerosis	373	1.6	4.8	1.8	1.1
	Deinococcus radiodurans	35073	2.0	1.6	2.1	0.8
	Derxia gummosa	15994	3.0	1.5	1.7	1.8
	Gardnerella vaginalis	14018	2.0	2.2	1.3	1.2
	Hafnia alvei	13337	1.0	2.5	1.7	1.6
20	Lactobacillus acidophilus	4356	2.0	2.7	2.0	1.9
	Moraxella osloensis	19976	2.0	2.1	1.9	1.8
	Mycobacterium smegmatis	14468	1.6	1.8	1.8	1.7
	Mycoplasma hominis	14027	1.5	1.8	1.6	1.5
	Neisseria gonorrhoeae	19424	2.0	2.7	1.6	1.6
25	Rahnella aquatilis	33071	2.0	2.7	2.3	2.1
	Rhodospirillum rubrum	11170	2.0	1.8	1.6	1.5
	Vibrio parahaemolyticus	17802	2.5	3.1	1.7	1.6
	Yersinia enterocolitica	9610	2.0	1.8	2.3	2.2
30	Human		2.0	1.8	2.1	3.0

Two derivatives of probe 1 also were made:

CCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAAC

CCCGACCGTCCCTATTAATCATTACGATGG

The first derivative works well at 65°C, the second at 60°C.

Example 21

Gonorrhea is one of the most commonly reported bacterial infections in the United States, with over two million cases reported annually. This sexually transmitted disease usually results in anterior urethritis in males and involves the cervix in females. While severe complications and even sterility

can occur in untreated individuals, asymptomatic infections are common, resulting in carriers who unknowingly spread the disease.

The causative agent, Neisseria gonorrhoeae, is a gram negative, oxidase positive diplococcus with stringent growth requirements. The method used for diagnosis depends on the site of infection and the patient symptoms. Gonococcal urethritis in males is diagnosed with good sensitivity and specificity using gram stain. Culture, requiring 24-72 hours, usually must be performed to confirm diagnosis of gonorrhea from all females and asymptomatic males. Following the detection of the organism from growth in culture, Neisseria gonorrhoeae must be identified by further tests such as carbohydrate degradation, coagglutination, fluorescent antibody screens or chromogenic enzyme substrate assays.

Neisseria gonorrhoeae is particularly difficult to detect and distinguish using a nucleic acid probe because it is very closely related to N. meningitidis. Data published in Kingsbury, D.T., J. Bacteriol. 94:870-874 (1967) shows a DNA:DNA homology for the two species of approximately 80-94%. Under guidelines established by the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics, Int'l J. System. Bacteriol. 37:463-464 (1987), the phylogenetic definition of a species generally means 70% or greater DNA:DNA homology. Despite the fact that these organisms may be considered to be the same species under established principles, we were able to make probes capable of distinguishing them.

As expected, the rRNA homology between N. gonorrhoeae and N. meningitidis is even greater because of known conserved regions. We noted a 1.0% difference between the 16S

and a 1.1% difference between the 23S rRNA sequences of N. gonorrhoeae and N. meningitidis using our sequencing data.

Making a probe for N. gonorrhoeae was complicated by the fact that in some sites where N. meningitidis and N. gonorrhoeae differed, other Neisseria species were similar to N. gonorrhoeae. The few mismatches which exist between these two species are in the most variable regions, i.e., regions which vary not only between species, but also from strain to strain. Despite the fact that some believed the species could not be distinguished with nucleic acid probes at all, and others believed that rRNA was too conserved to be useful in probe diagnostics, we were able to make probes capable of differentiating N. gonorrhoeae and N. meningitidis.

The present invention has significant advantages over each of the prior art methods; the probes are more specific and much faster than culture methods. It also is believed that the probes are more sensitive, (i.e., able to detect a smaller number of organisms in a clinical sample) than prior art methods.

The primers used to identify these probe sequences had the following sequences:

1. GGCCGTTACCCACCTACTAGCTAAT
2. GTATTACCGCGGCTGCTGGCAC
3. GCTCGTTGCGGGACTTAACCCACCAT

Each of the rRNA sites chosen to target had at least two mismatches to E. coli, N. meningitidis, N. cinerea, N. lactamica, N. mucosa, and Kingella kingae.

Oligonucleotides complementary to sequences adjacent to the probe regions were synthesized and used in the hybridization mix according to Hogan et al., U.S. Patent App. Ser. No.

134 ~~(not yet assigned), to be filed November 24, 1987,~~
entitled "Means and Method for Enhancing Nucleic Acid
Hybridization (the "helper" patent application).

5 The following sequences were characterized and shown to
be specific for Neisseria gonorrhoeae. The phylogenetically
nearest neighbors Neisseria meningitidis, N. lactamica, N.
cinerea, N. mucosa, and Kingella kingae were used for comparison
with the N. gonorrhoeae sequence.

1. CCG CCG CTA CCC GGT AC
- 10 2. TCA TCG GCC GCC GAT ATT GGC
3. GAG CAT TCC GCA CAT GTC AAA ACC AGG TA

Sequence 1, complementary to 16S rRNA in the region 125-150, is
17 bases in length and has a T_m of 56°C. Sequence 2,
complementary to 16S rRNA in the region 455-485, is 21 bases in
15 length and has a T_m of 63°C. Sequence 3, complementary to 16S
rRNA in the region 980-1015, is 29 bases in length and has a T_m
of 57°C.

The reactivity and specificity of the probes for
Neisseria gonorrhoeae was demonstrated with a hybridization
20 assay. The three oligonucleotide probes were iodinated and mixed
with unlabeled oligonucleotides of sequence 5'-CCC CTG CTT TCC
CTC TCT AGA CGT ATG CGG TAT TAG CTG ATC TTT CG-3', 5'-GCC TTT TCT
TCC CTG ACA AAA GTC CTT TAC AAC CCG-3', 5'-GGC ACG TAG TTA GCC
GGT GCT TAT TCT TCA GGT AC-3', and 5'-GGT TCT TCG CGT TGC ATC GAA
25 TTA ATC CAC ATC ATC CAC CGC-3', and with purified RNA in 0.48 M
sodium phosphate, ^{pH 6.8} ~~pH 6.8~~, 0.5% sodium dodecyl sulfate (SDS) and
incubated at 60°C for one hour. Following incubation, 4 ml of 2%
hydroxyapatite, ^{pH 6.8} ~~pH 6.8~~, 0.02% SDS was
added and the mixture was incubated at 60°C for 5 minutes. The

13 samples were centrifuged and the supernatants were removed. Five ml of wash solution (0.12 M sodium phosphate ^{pH 6.8} ~~pH 6.8~~, 2% SDS) was added and the samples were mixed, centrifuged, and the supernatants removed. The amount of radioactivity bound to the hydroxyapatite was determined in a gamma counter.

Table 72 shows that the probes hybridize well to *N. gonorrhoeae* RNA and do not hybridize to the other species tested.

TABLE 72

HYBRIDIZATION OF *NEISSERIA GONORRHOEAE*
PROBES 1-3 TO *NEISSERIA* AND *KINGELLA* RNAS

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Kingella kingae	23332	0.09
	Neisseria cinerea	14685	0.04
15	N. gonorrhoeae	19424	48.4
	N. lactamica	23970	0.07
	N. meningitidis serogroup A	13077	0.04
	N. meningitidis serogroup B	13090	0.04
	N. meningitidis serogroup C	13102	0.04
20	N. mucosa	19696	0.07
	N. subflava	14799	0.05

The following derivatives of *Neisseria* probes also have been made and used:

25 GAG GAT TCC GCA CAT GTC AAA ACC AGG
GAG GAT TCC GCA CAT GTC AAA ACC AGG TAA
CCC GCT ACC CGG TAC GTT C
CCG CTA CCC GGT ACG TTC.

30 Although the above examples of performance were determined using the standard assay format previously described, the specific probes may be used under a wide variety of

experimental conditions. For example, additives may be included to the reaction solutions to provide optimal reaction conditions for accelerated hybridization. Such additives may include buffers, chelators, organic compounds and nucleic acid precipitating agents such as detergents, dihydroxybenzene, sodium dodecyl sulfate, sodium diisobutyl sulfosuccinate, sodium

tetradecyl sulfate, sarkosyl and the alkali metal salts and ammonium salts of ^{SO₄²⁻, PO₄³⁻, Cl⁻ and HCOO⁻.} ~~SO₄²⁻, PO₄³⁻, Cl⁻ and HCOO⁻~~ Such additives

can be utilized by one skilled in the art to provide optimal conditions for the hybridization reaction to take place. These conditions for accelerated hybridization of single stranded nucleic acid molecules into double stranded molecules are the subject of the above-noted ^{U.S. Patent 5,132,207.} ~~U.S. Patent App. Ser. No. 627,795,~~

~~filed July 5, 1984, continuation filed June 4, 1987 (serial no. not yet assigned) and Ser. No. 816,711 filed January 7, 1986, which are both entitled ACCELERATED NUCLEIC ACID REASSOCIATION METHOD.~~

The present invention can be carried out on nonviral organisms from purified samples or unpurified clinical samples such as sputum, feces, tissue, blood, spinal or synovial fluids serum, urine or other bodily fluids, or other samples such as environmental or food samples. Prior to cell breakage and hybridization, the cells can be suspended or placed in solution. In the case of the unpurified samples referred to above, the cells may remain intact and untreated in their own biological environment prior to the assay.

The probes of the present invention may be used in an assay either alone or in combination with different probes. Several individual probes also can be linked together during

nucleic acid synthesis. This results in one probe molecule which contains multiple probe sequences, and therefore, multiple specificities. For example, a single nucleic acid molecule can be synthesized which contains both the Mycobacterium avium and
5 the Mycobacterium intracellulare sequences described in Examples 1 and 2. When hybridized with either M. avium or M. intracellulare rRNA this probe will hybridize completely. If the two probe sequences were combined separately in an assay only one half of the mixed individual probes will hybridize with either
10 M. avium or M. intracellulare rRNA. Other embodiments also may be practiced within the scope of the claims. For example, probes may be labelled using a variety of labels, as described within, and may be incorporated into diagnostic kits.